Characterization of Biomarkers of Immunological Activation in African Elephants (*Loxodonta africana*)

by

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Declaration

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

This dissertation includes a general introduction (Chapter 1), one original paper published in Cytokine, a peer-reviewed journal (Chapter 2), one chapter containing unpublished work (Chapter 3), a general discussion (Chapter 4) and conclusion (Chapter 5). The development and writing of this thesis (published and unpublished) were the principal responsibility of myself.

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Abstract

African elephants (*Loxodonta africana*) are considered priority species within conservation areas because of their aesthetic value, ecological importance, and economic contribution to the ecotourism industry. Conservation efforts have focused on protecting habitat, but there are few studies investigating the role of disease. The recent discovery of tuberculosis (TB) in a free-ranging African elephant in Kruger National Park (KNP), South Africa, has resulted in movement restrictions, preventing the translocation of elephants from this population. Since diagnostic tests for TB in wildlife are limited, the development of blood-based tests to detect *Mycobacterium tuberculosis* complex (MTBC) infection in African elephants is needed. These antigen-specific immune assays would have a significant beneficial impact on current practices in wildlife and zoological medicine. Therefore, the aim of this project was to identify blood-based host biomarkers that can be used to detect immune responses of African elephants.

Cytokine gene expression assays (GEAs) have been employed to measure cell-mediated immune responses in a variety of species. These GEAs use real-time, reverse-transcription quantitative PCR (RT-qPCR) to measure changes in gene expression of immune cells, following stimulation of whole blood. In this study, whole blood from African elephants from KNP, a *Mycobacterium bovis*-endemic area, was stimulated using pokeweed mitogen and mycobacterial antigens. Newly designed primers, as well as modified primers originally developed for use in other species, were used to amplify and sequence African elephant mRNA transcripts of selected target (*CXCL9, CXCL10, IFNγ, IL4, IL10, IL12, TGFβ*, and *TNF*) and reference genes (*ACTB*, *B2M*, *GAPDH*, *YWHAZ*). These mRNA transcripts were used to design sequence specific primers and develop a RT-qPCR to determine changes in cytokine expression as a measure of general immune activation and antigen-specific responses.

Confirmed mRNA transcripts for African elephants were used to develop real-time RT-qPCRs for *IL10*, *TNF*, and *TGFβ*, relative to *GAPDH* as the optimal reference gene. These cytokine GEAs demonstrated the use of identified biomarkers to measure immune responses in this species. To our knowledge, this was the first study that has investigated cytokine biomarkers in African elephants using real-time RT-qPCR.

Results of the cytokine GEAs showed up-regulation of *IL10* and *TNF*, as well as down-regulation of *TGFβ*, in response to mitogen stimulation. When expression of these cytokines was evaluated in response to mycobacterial antigen stimulation, a significant up-regulation of *IL10* was observed following PPDa and PPDb stimulation. However, following stimulation

with ESAT6/CFP10, as well as calculated differential PPD response, a very slight down-regulation of *IL10* was observed, as expected in TB uninfected elephants. Similarly, a slight down-regulation of *TNF* and *TGFβ* was observed following all mycobacterial antigen stimulations.

Findings in this study provide novel insights into the African elephant immune system. The generated mRNA transcripts provide a basis for development of immunological assays for TB, as well as other diseases. Finally, evaluation of gene expression following antigen stimulation provided insight into the use of PPDa, PPDb and ESAT6/CFP10 as stimulants of antigen-specific TB responses. This will aid in the development of tools to improve disease detection and diagnosis in African elephants.

Opsomming

Afrika-olifante (*Loxodonta africana*) word as ’n prioriteitspesies in bewaringsgebiede beskou, vanweë hul estetiese waarde, ekologiese belang en ekonomiese bydrae tot ekotoerisme bedrywighede. Bewaringspogings fokus grotendeels op die beskerming van habitat, maar daar is 'n slegs n paar studies wat die rol van siektes in hierdie spesie ondersoek. Die onlangse ontdekking van tuberkulose (TB) in 'n vrylopende Afrika-olifant in die Krugerwildtuin (KNP), Suid-Afrika, het gelei tot bewegingsbeperkings wat die skuif van Afrika-olifante uit hierdie bevolking verhinder. Aangesien diagnostiese toetse vir TB in wildsoorte beperk is, is die ontwikkeling van 'n bloedtoets om infeksie met *Mycobacterium tuberculosis* kompleks (MTBC) in Afrika-olifante op te spoor nodig. Antigeen-spesifieke immuuntoetse sal 'n voordelige impak op die bestaande praktyke in wild- en dierkundige medisyne hê. Die doel van hierdie projek was dus om bloedgebaseerde gasheer-biomerkers te identifiseer wat gebruik kan word om immuunresponse van Afrika-olifante te meet.

Sitokien geenuitdrukkings-toetse (GEA’s) is voorheen gebruik om sel-gemedieerde immuunresponse in 'n verskeidenheid spesies te meet. Hierdie toetse gebruik ware-tyd, omgekeerde-transkripsie kwantitatiewe polimerase kettingreaksie (RT-qPCR) om veranderinge in geenuitdrukking in immuunselle, in gestimuleerde volbloed te meet. In hierdie studie is volbloed van Afrika-olifante vanuit KNP, ‘n *Mycobacterium bovis* endemiese area, deur middel van ‘n ‘pokeweed’ mitogeen asook mikobakteriële antigene gestimuleer. Nuut ontwerpte inleiers, asook gemodifiseerde inleiers wat oorspronklik vir ander spesies ontwerp was, is gebruik om mRNA-transkripte van geselekteerde teiken- (*CXCL9, CXCL10, IFNγ, IL4, IL10, IL12, TGFβ* and *TNF*) en verwysingsgene (*ACTB, B2M, GAPDH, YWHAZ*) van Afrika-olifant te amplifiseer en die volgorder te bepaal. Hierdie mRNA-transkripte was toe gebruik om spesifieke sitokien inleiers te ontwerp en 'n RT-qPCR te ontwikkel om veranderinge in sitokienuitdrukking te bepaal om sodoende algemene immuunaktivering en antigeenspesifieke reaksies te meet.

Bevestigde mRNA-transkripte vir Afrika-olifante is gebruik om ware-tyd RT-qPCR vir *IL10, TNF* en *TGFβ* te ontwikkel, relatief tot *GAPDH* as die optimale verwysingsgeen. Hierdie sitokien GEAs dui op die moontlike gebruik van die geïdentifiseerde biomerkers om immuunresponse in hierdie spesie te meet. Na ons wete was dit die eerste studie wat sitokien-biomerkers in Afrika-olifante ondersoek het deur gebruik te maak van ware-tyd RT-qPCR.

Resultate van die sitokien-GEA's het ’n toename in die *IL10* en *TNF* geenuidrukking asook ’n afnameing in *TGFβ* geenuitdrukking getoon nadat volbloed met mitogeen gestimuleer was. Toe die uitdrukking van hierdie sitokiene na mikobakteriële antigeenstimulasie evalueer was, het die uitdrukking van *IL10* beduident toegeneem na PPDa- en PPDb-stimulasie. Na stimulasie met ESAT6/CFP10, sowel as berekende differensiële PPD-response, is afwaartse regulering van *IL10* egter waargeneem, in onbesmette olifante. Net so is afname in *TNF* en *TGFβ* geenuitdrukking waargeneem na aanleiding van mikobakteriële antigeensimulasies.

Bevindings in hierdie studie bied nuwe insigte tot die immuunsisteem van die Afrika-olifant. Die gegenereerde mRNA-transkripte bied 'n basis vir die ontwikkeling van immunologiese toetse, nie net vir TB nie, maar ook vir ander siektes. Laastens bied hierdie ondersoek in geenuitdrukking na antigeenstimulasie insig oor die gebruik van PPDa, PPDb en ESAT6/CFP10 as aanduiders van antigeenspesifieke TB-reaksies. Dit sal help met die ontwikkeling van instrumente om die opsporing en diagnose van siektes in Afrika-olifante te verbeter.

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List of symbols and abbreviations

°C	degree Celsius
ΔCq	delta Cq
ΔΔCq	delta delta Cq
µl	microliter
$2^{-\Delta\Delta Cq}$	fold change
ACTB	actin-beta
B2M	beta-2-microglobulin
BAL	bronchoalveolar lavage
bp	base pairs
cDNA	complementary DNA
CFP10	culture filtrate protein 10 kDa
CMI	cell-mediated immune
ConA	concanavalin A
Cq	quantification cycle
CXCL9	C-X-C motif chemokine ligand 9
CXCL10	C-X-C motif chemokine ligand 10
DALRRD	Department of Agriculture, Land Reform and Rural Development
DM	derivative melt curve peak
DNA	deoxyribonucleic acid
DPP®	Dual Path Platform
E	amplification efficiency
EEHV	elephant endotheliotropic herpesviruses
ELISA	enzyme-linked immunosorbent assay
ESAT6	early secretory antigenic target 6 kDa
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GCs	glucocorticoids
gDNA	genomic DNA
GEAs	gene expression assays
IFNγ	interferon-gamma
IGRAs	interferon-γ release assays
IAV	intra-assay variability
IL4	interleukin 4
IL10	interleukin 10
IL12	interleukin 12

IUCN	International Union for Conservation of Nature
kDa	kilodaltons
KNP	Kruger National Park
NK	natural killer
ng	nanogram
nM	nanomoles
M. bovis	*Mycobacterium bovis*
mL	milliliter
mRNA	messenger RNA
M. tb	*Mycobacterium tuberculosis*
MTBC	*Mycobacterium tuberculosis* complex
OIE	World Organization for Animal Health
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PHA	phytohaemagglutinin
PPDa	avium purified protein derivative
PPDb	bovine purified protein derivative
PWM	pokeweed mitogen
QFT	QuantiFERON® TB Gold Plus
QFT-TB2	QuantiFERON®-TB Gold TB2
RD	region of difference
RNA	ribonucleic acid
rRNA	ribosomal RNA
RT-qPCR	reverse-transcription quantitative PCR
SANBI-NZG	South African National Biodiversity Institute-National Zoological Gardens
SCITT	single comparative intradermal tuberculin test
SNP	single nucleotide polymorphism
TB	tuberculosis
TGFβ	transforming growth factor beta
TNF	tumor necrosis factor
USA	United States of America
USAHA	United States Animal Health Association
USDA	United States Department of Agriculture
YWHAZ	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta

List of publications

Candice R. de Waal, Léanie Kleynhans, Sven D.C. Parsons, Wynand J. Goosen, Guy Hausler, Peter E. Buss, Robin M. Warren, Paul D. van Helden, Jennifer A. Landolfi, Michele A. Miller, and Tanya J. Kerr. (2021). Development of a Cytokine Gene Expression Assay for the Relative Quantification of the African Elephant (*Loxodonta africana*) Cell-Mediated Immune Responses. **Published:** Cytokine, 141(155453), pp. 1–8. doi: 10.1016/j.cyto.2021.155453.

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Chapter 1: General Introduction

1.1. Background

1.1.1. African elephants

African elephants (*Loxodonta africana*) are listed as vulnerable on the International Union for Conservation of Nature's (IUCN) Red List of Threatened Species (Blanc, 2008). This is due to factors such as ongoing poaching and illegal hunting (Schlossberg, Chase and Sutcliffe, 2019). Other factors that contribute to declining African elephant populations include habitat reduction and fragmentation (Sach *et al.*, 2019), which lead to increased human-animal conflict (Mmbaga, Munishi and Treydte, 2017; de Sales, Anastácio and Pereira, 2020). While research and conservation efforts have focused primarily on habitat protection (Branco *et al.*, 2019; Sach *et al.*, 2019), there is still a knowledge gap relating to the role of infectious diseases, such as elephant endotheliotropic herpesviruses (EEHV) and tuberculosis (TB), in African elephant populations (Maslow and Mikota, 2015; Bronson *et al.*, 2017; Abegglen *et al.*, 2018).

1.1.2. *Mycobacterium tuberculosis* complex (MTBC) in African elephants

The *Mycobacterium tuberculosis* complex (MTBC) consists of various members of closely related gram-positive, acid-fast bacteria, which are primarily transmitted via aerosols (Fowler, 2008; Coscolla and Gagneux, 2014). Of these, *Mycobacterium tuberculosis* (*M. tb*) is most commonly found in African and Asian elephants (*Elephas maximus*) (Mikota *et al.*, 2001; Miller *et al.*, 2019), with a few cases of *Mycobacterium bovis* (*M. bovis*) (Lyashchenko *et al.*, 2006; Goosen *et al.*, 2020a). Tuberculosis in elephants was identified as an emerging disease following the death of several captive Asian elephants in the 1990's (Mikota *et al.*, 2001) and since then, the disease has been found in various managed elephant populations across the world (Lewerin *et al.*, 2005; Mikota, 2008; Angkawanish *et al.*, 2010; Verma-Kumar *et al.*, 2012). Although many of the reported cases have been in captive Asian elephants, TB has also been documented in free-ranging Asian elephants in Sri Lanka and India (Perera *et al.*, 2014; Chandranaik *et al.*, 2017; Zachariah *et al.*, 2017). In 2013, the first fatal case of TB in a free-ranging African elephant was discovered in Kenya (Obanda *et al.*, 2013). More recently, a fatal case was discovered in an African elephant bull in Kruger National Park (KNP), South Africa (Miller *et al.*, 2019).

Tuberculosis in elephants is likely an anthropozoonotic disease (Zachariah *et al.*, 2017; Lainé, 2018). An isolate of *M. tb* from an Asian elephant in Nepal shared a synonymous single nucleotide polymorphism (SNP) with the isolate found in two human samples in the region (Paudel *et al.*, 2014), indicating possible spillover from a local handler. Elephant-human contact is a frequent occurrence in elephants housed in zoos and used as service animals in temples and logging camps. Indirect contact may also occur with people that enter protected areas and national parks. Thus, disease spillover from humans to elephants is a rising One Health concern (Lainé, 2018; Rosen *et al.*, 2018).

1.1.3. MTBC diagnostics in elephants

Tuberculosis in elephants is a chronic disease associated with nonspecific clinical signs of weight loss, anorexia, weakness, exercise intolerance, and/or abnormal discharge (Mikota *et al.*, 2001; Mikota and Maslow, 2011; Paudel and Toshio, 2016). Signs are typically observed only with advanced stages of infection, and often TB is only diagnosed at necropsy (Mikota and Maslow, 2011; Paudel and Toshio, 2016). Thus, the first step in managing the spread and impact of TB in elephants is early detection. Unlike humans, there are limited options available for the diagnosis of TB in elephants. Current diagnostic tests for TB in elephants can be classified into two categories: direct detection of the MTBC organisms, and indirect detection by measuring cell-mediated or humoral immune responses to mycobacterial antigens. Direct detection of the MTBC organisms is achieved using mycobacterial culture and polymerase chain reaction (PCR) based methods. Indirect detection of MTBC infection, via cell-mediated and humoral immune responses, can be done *in vivo* or *in vitro*. The most common methods of MTBC diagnostics are discussed below.

1.1.3.1. Mycobacterial culture and PCR

Mycobacterial culture of respiratory samples, such as trunk wash (TW) and bronchoalveolar lavage (BAL) fluid, is currently regarded as the "gold standard" for antemortem TB diagnosis in elephants (World Organisation for Animal Health (OIE), 2018). The United States Department of Agriculture (USDA) has recommended the triple trunk wash method for annual TB screening (Mikota et al., 2001; United States Animal Health Association (USAHA) Elephant Tuberculosis Subcommittee, 2010). With this method, trained elephants aspirate sterile saline into their trunks and then forcibly expel it, and this fluid is collected into a sterile container. In other cases, the trunk is held up by the trainer and sterile saline is poured into the nares to aid in the recovery of respiratory secretions. Samples are collected on three separate

days, within a seven-day period, and transported to a laboratory that is equipped for mycobacterial culture. Unfortunately, mycobacterial culture of TW samples has certain drawbacks. Mycobacterial culture has varying sensitivity, especially since elephants shed mycobacteria intermittently (Fowler, 2008; Maslow and Mikota, 2015; Paudel and Toshio, 2016). Thus, while a positive culture can confirm TB, a negative culture cannot rule out infection. Samples are often difficult to collect, as elephants need to be immobilized or trained for sample collection. Consecutive sample collection is difficult to achieve in free-ranging elephants since it requires repeated immobilizations. Elephants also use their trunks to explore their environment and collect food, increasing the likelihood of contamination with environmental bacteria (Hermes *et al.*, 2018). Alternative methods of sample collection have also been explored. A study by Goosen *et al.* (2020a) described a modified procedure for collecting trunk wash samples from free-ranging African elephants. A single TW sample (approximately 250 mL) is collected from each nostril, separately, during immobilization and aspirated into a sterile 500 mL collection chamber. In addition, BAL samples, collected using a flexible endoscope inserted via the oral cavity of immobilized elephants Goosen *et al.* (2020a), or via the trunk of trained captive elephants (Hermes *et al.*, 2018) have been used as alternative methods to collect respiratory samples for mycobacterial culture.

Mycobacterial culture is a slow and costly procedure which requires processing in a BSL3 facility, and 6-8 weeks of incubation. Additionally, culture-positive samples require further speciation using methods such as region of difference (RD) PCR, 16S rRNA sequencing, and spoligotyping to identify mycobacterial strains (Kamerbeek *et al.*, 1997; Leclerc *et al.*, 2000; Warren *et al.*, 2006). Two alternative PCR-based methods have been used for the direct detection of MTBC organisms in African elephant samples, including the GeneXpert® MTB/RIF Ultra Assay (Cepheid, Sunnyvale, CA, USA) that relies on detection of insertion elements IS6110 and IS1081, and the VetMAX™ MTBC qPCR kit that detects the insertion element IS6110 (Goosen *et al.*, 2020a, 2020b). The advantage of direct PCR is that it provides more rapid results and may be more sensitive for detecting organisms in paucibacillary samples.

1.1.3.2. Intradermal tuberculin test

The intradermal tuberculin test is the only *in vivo* test recommended by the OIE, for the detection of TB in cattle (Cousins and Florisson, 2005). The single comparative intradermal tuberculin test (SCITT) has been used to detect TB in wildlife species such as African buffaloes (*Syncerus caffer*), African lions (*Panthera leo*), and common warthogs (*Phacochoerus africanus*) as well as many wildlife species in zoos (Keet *et al.*, 2010; Miller and Lyashchenko, 2015; Bernitz *et al.*, 2018b; Roos *et al.*, 2018). However, the SCITT has proven to be unreliable in pachyderms such as elephants, with a low sensitivity (16.7%) and specificity (72.4%) (Mikota *et al.*, 2001), and therefore is not recommended for TB diagnosis in elephants (Miller and Lyashchenko, 2015).

1.1.3.3. Serology

Various mycobacterial antigens have been identified that elicit antibody responses in the host following exposure. The most common antigens used for diagnostics in elephants include the lipoproteins MPT70 and MPT83 (MPB70 and MPB83 in *M. bovis*) and the MTBC-specific antigens, early secretory antigenic target 6 kDa (ESAT6) and culture filtrate protein 10 kDa (CFP10) (Lyashchenko *et al.*, 2006; Greenwald *et al.*, 2009). Along with mycobacterial culture, the USDA has recommended the use of the Dual Path Platform (DPP®) Vet TB Assay for Elephants (Chembio Diagnostic Systems, Inc., Medford, NY, USA) as part of annual TB surveillance in captive elephants (USAHA Elephant Tuberculosis Subcommittee, 2010). This commercially available assay uses a lateral-flow design, to deliver whole blood, serum, or plasma to a nitrocellulose strip containing a control line and two test lines that detect MTBC antigen-specific IgG antibodies. The first test line contains the antigen MPB83, while the second contains the fusion protein ESAT6/CFP10 (Greenwald *et al.*, 2009). The results are read using the DPP® optical-reader device or visually, with a visible line at either or both test lines considered positive for TB antibodies (Greenwald *et al.*, 2009).

Elephants develop a strong humoral response to TB, with serodiagnosis sometimes made years before there is a positive mycobacterial culture result (Lyashchenko *et al.*, 2006; Greenwald *et al.*, 2009). High sensitivity (approaching 100%) and specificity (approaching 95%) of serological assays have been demonstrated in captive African and Asian elephants using the ElephantTB STAT-PAK® and DPP® Vet TB Assay (Greenwald *et al.*, 2009; Lyashchenko *et al.*, 2012, 2018). A retrospective study by Kerr *et al.* (2019) also showed the utility of these assays for serosurveillance in a free-ranging African elephant population. It is

however important to note that a positive serological result does not equate with current infection, since detection of antibodies can also indicate immunity to previous infections, or cross-reactivity with shared antigens from other pathogens, such as *Mycobacterium szulgai* (Lacasse *et al.*, 2007; Greenwald *et al.*, 2009). Additionally, both false positive and false negative results in elephants have been obtained with serological testing (Lacasse *et al.*, 2007; Greenwald *et al.*, 2009).

1.1.3.4. Interferon-γ release assays (IGRAs)

Cell-mediated immune (CMI) responses are elicited in many species during early infection with *M. tb* or *M. bovis* (Thoen and Barletta, 2006; Zuniga *et al.*, 2012). These responses can be measured *in vitro* by cytokine protein production, using species-specific enzyme-linked immunosorbent assays (ELISAs) after antigen stimulation of whole blood. One of the cytokines that has been shown to be important in CMI responses to TB is interferon-gamma (IFNγ) (Cavalcanti *et al.*, 2012; Romero-Adrian *et al.*, 2015). Interferon-γ release assays (IGRAs) have been used to diagnose TB in humans as well as various animal species including cattle (*Bos taurus*), domestic cats (*Felis catus*), African buffaloes, wild dogs (*Lycaon pictus*), white rhinoceros (*Ceratotherium simum*), and black rhinoceros (*Diceros bicornis*) (Rhodes *et al.*, 2011; Bass *et al.*, 2013; Pari *et al.*, 2014; Miller and Lyashchenko, 2015; Bernitz *et al.*, 2018a). Cytokine release assays such as IGRAs, often require species-specific antibodies, or antibodies from a closely related species, with high identity between protein sequences. For example, the ruminant Cattletype® IFNγ ELISA (INDICAL, Inc., San Francisco CA, USA) originally designed for cattle, has been successfully used to detect African buffalo IFNγ (Bernitz *et al.*, 2018a). In species where compatible reagents are not commercially available, the development and validation of these assays can be expensive and time consuming (Abegglen *et al.*, 2018). Three IGRAs have been developed for the diagnosis of TB in elephants, however, whole blood from only a single African elephant was available, and validation was performed using Asian elephant samples (Angkawanish *et al.*, 2013; Paudel *et al.*, 2016; Songthammanuphap *et al.*, 2020). Further validation in African elephant populations is thus required.

1.1.3.5. Cytokine gene expression assays (GEAs)

As an alternative to cytokine release assays, cytokine gene expression assays (GEAs) have been employed to measure antigen-specific CMI responses. These GEAs use real-time, reverse-transcription quantitative PCR (RT-qPCR) to measure changes in gene expression of immune cells following antigen stimulation of whole blood. The use of real-time RT-qPCR provides a rapid and cost-effective method to determine infection status using antemortem samples (VanGuilder, Vrana and Freeman, 2008; Derveaux, Vandesompele and Hellemans, 2010). For the most part, reagents for RT-qPCR are commercially available, while online tools facilitate primer design using species-specific cytokine sequences which are relatively conserved in mammals (VanGuilder, Vrana and Freeman, 2008; Roos *et al.*, 2019).

Cytokine GEAs have been used to diagnose mycobacterial infection in various wildlife species such as Asian elephants, spotted hyenas (*Crocuta crocuta*), African lions, and common warthogs (Landolfi *et al.*, 2009; Higgitt *et al.*, 2017; Olivier *et al.*, 2017; Roos *et al.*, 2019). Landolfi *et al.* (2009) developed real-time RT-qPCRs for various cytokines involved in humoral and cell-mediated immune responses for use in Asian elephants. A follow-up study highlighted trends in expression of certain circulating cytokines that differed between TB seronegative and seropositive elephants (Landolfi *et al.*, 2010). Finally, the cytokine GEAs were used to categorize elephant infection status using antigen stimulated peripheral blood mononuclear cell (PBMC) cultures from *M. tb* positive and negative Asian elephants (Landolfi *et al.*, 2014). The high level of sequence identity between Asian and predicted African elephant mRNA sequences (generated using computational analysis) further highlights the potential utility of cytokine GEAs for the detection of TB in African elephants (Benson *et al.*, 2013).

1.2. Problem statement

The recent discovery of TB in a free-ranging African elephant in KNP has resulted in movement restrictions imposed by the South African Department of Agriculture, Land Reform and Rural Development (DALRRD), preventing the translocation of African elephants from this population (Miller *et al.*, 2019). In addition, *M. tb* infection contributed to the cause of death in two captive elephants in the South African National Biodiversity Institute National Zoological Gardens (SANBI-NZG) (unpublished data). Due to the ecological and economical importance of this species for the to the ecotourism industry in South Africa (Blignaut, de Wit and Barnes, 2008; Kerley *et al.*, 2008), investigating the role of TB in African elephants is crucial for conservation. Since diagnostic tests for TB in wildlife are limited, especially in

elephants, the development of a blood-based test to detect MTBC infection in African elephants using antigen-specific immunoassays would have a significant beneficial impact on current practices in wildlife and zoological medicine.

1.3. Significance and motivation

The impact of this research will be basic knowledge generation to provide a greater understanding of health in African elephants, a valued national asset for South Africa and the African continent. Application of immunological and molecular biological techniques will produce new information regarding health and disease in these long-lived mammals, which may serve as a model for comparative biology to humans and other long-lived species. In addition, the biomarkers identified will be useful for further development of diagnostic tools for disease detection and surveillance in African elephants, as well as individual health assessment and monitoring. This is important for improving welfare and management of captive individuals but also for determining the impact of disease in free-ranging populations. A crucial component of conservation programs is to ensure that only healthy animals are translocated due to the stress on the individual, costs, logistics, and potential outcome of the move for the success of the program. Therefore, diagnostic tools which could minimize potential disease introduction during translocation of animals between fragmented populations is needed.

Since diagnostic tests for TB in wildlife are limited, especially in elephants, the development of a blood-based test to detect infection in individual African elephants and for surveillance in populations, using antigen-specific immunoassays, would have a significant impact on current practices in wildlife medicine. Providing accurate tools for disease detection to veterinarians, wildlife managers, and decision-makers will enhance individual and population management, and better inform policies affecting the health of the species.

1.4. Aims and Objectives

Based on the documented reports of TB in African elephants in KNP, antigen-specific immunological responses in this population were investigated using whole blood stimulated with mycobacterial peptides. The overall goal of this project was to generate new tools for assessing responses to TB, an infectious disease that impacts the health of African elephants (both free-ranging and captive populations). These tools will improve disease detection and diagnosis, as well as advance our understanding of disease pathogenesis and epidemiology for overall improvement of welfare and conservation of this species.

Main Aim: To identify blood-based host biomarkers that can be used to detect immune responses of African elephants.

Aim 1: Develop and optimize a cytokine gene expression assay (GEA) to measure immune activation in African elephants, using mitogen stimulated whole blood.

- Objective 1.1: To amplify full-length African elephant mRNA transcripts for cytokine targets identified as potential biomarkers for TB from literature.
- Objective 1.2: To identify an optimal reference gene using African elephant mitogen stimulated whole blood.
- Objective 1.3: To determine diagnostic cytokine target genes to measure immune activation in African elephants.
- Objective 1.4: To identify optimal incubation time for mitogen stimulation of African elephant whole blood to measure target gene expression.
- Objective 1.5: To identify potential biomarkers of immune activation, using the developed cytokine GEA.

Aim 2: Characterize changes in cytokine gene expression in African elephants using antigen stimulated whole blood.

- Objective 2.1: Determine the differences in gene expression between TB seropositive- and negative African elephants, using the cytokine GEA developed as part of Aim 1.

1.5. Thesis overview

Chapter 1: General Introduction

This chapter highlights the significance and potential impact of the study by providing a brief background on *M. tb*, as well as the impact and history of the disease in African elephants. The overall research theme is outlined in this chapter, including study aims and objectives.

Chapter 2: Development of a cytokine gene expression assay for the relative quantification of the African elephant (Loxodonta africana) cell-mediated immune responses

This chapter describes the development of a real-time, reverse-transcription quantitative PCR (RT-qPCR) assay for use with RNA extracted from African elephant whole blood. This includes the generation of novel, full-length and partial cytokine mRNA transcripts from pokeweed mitogen (PWM) stimulated African elephant whole blood. A panel of reference and immune mediator target genes was evaluated to identify candidate biomarkers of *in vitro* immune activation in African elephants to advance the understanding of African elephant specific immune responses. Four reference genes were evaluated: actin-beta (*ACTB*), beta-2-microglobulin (*B2M*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (*YWHAZ*). A panel of eight immune mediator target genes was also examined: C-X-C motif chemokine ligands 9 (*CXCL9*) and 10 (*CXCL10*); interleukins 4 (*IL4*), 10 (*IL10*) and 12 (*IL12*); interferon-gamma (*IFNγ*), transforming growth factor beta (*TGFβ*), and tumor necrosis factor (*TNF*). The optimal reference gene (*GAPDH*) was identified, as well as three target genes (*IL10, TGFβ*, and *TNF*) that met the criteria needed to develop a RT-qPCR. This chapter has been published in Cytokine, a peer-reviewed journal.

Chapter 3: Evaluation of mycobacterial antigen stimulated responses in African elephant whole blood using cytokine gene expression assays

In this chapter, whole blood from African elephants (n = 11) were stimulated with ESAT6/CFP10, avium purified protein derivative (PPDa) and bovine purified protein derivative (PPDb) for 6 hours. The cohort contained ten MTBC uninfected African elephants based on serological and mycobacterial culture results, while one elephant was classified as MTBC seropositive, but mycobacterial culture negative. Following antigen stimulation, the ability of the optimized RT-qPCRs for target genes *IL10, TGFβ*, and *TNF*, normalized to the

reference gene *GAPDH*, was used to assess their potential use as biomarkers for TB in elephants.

Chapter 4: General discussion

This chapter provides a summary of our results and highlights the principle interpretations, with a focus on how these relate to the literature. In addition, limitations of the study are discussed.

Chapter 5: General conclusion and future research

In this chapter, the overall contribution of this study's findings to the understanding of immune responses in African elephants is highlighted. Recommendations for future research are also included.

Chapter 2: Development of a cytokine gene expression assay for the relative quantification of the African elephant (*Loxodonta africana*) cell-mediated immune responses

This chapter, published in the peer-revied journal *Cytokine*, describes the development of a real-time, reverse-transcription quantitative PCR (RT-qPCR) assay for use with RNA extracted from African elephant whole blood. This includes the generation of novel, full-length and partial cytokine mRNA transcripts from African elephant whole blood, as well as the identification of candidate biomarkers of *in vitro* immune activation in African elephants.

2.1. Introduction

African elephants (*Loxodonta africana*) are listed as vulnerable on the International Union for Conservation of Nature (IUCN) Red List of Threatened Species (Blanc, 2008). The main threats to elephant conservation are habitat fragmentation (Fowler, 2008) and increased human-animal conflict (Schlossberg, Chase and Sutcliffe, 2019); however infectious diseases also pose a threat and warrant further investigation. Importantly, infectious disease status needs to be considered when animals are translocated for conservation purposes (Fowler, 2008). Important infections affecting both human-managed and free-ranging Asian (*Elephas maximus*) and African elephant populations include elephant endotheliotropic herpesvirus (EEHV) and *Mycobacterium tuberculosis* (*M. tb*) (Fowler, 2008; Maslow and Mikota, 2015; Long, Latimer and Hayward, 2016). In both infections, the interplay between pathogen and the host immune response is an integral component of disease outcome and the focus of continued research to better understand pathogenesis. To date, research on immune responses and diseases has focused predominantly on Asian elephants, and information on African elephant immunology is sparse (Abegglen *et al.*, 2018).

Many indirect diagnostic tests are based on the host's immune response (Lyashchenko *et al.*, 2012; Clarke *et al.*, 2017; Chileshe *et al.*, 2019; Bernitz *et al.*, 2020a). To use antibody-based assays for assessment of immune responses in wildlife such as African elephants, species-specific reagents are often required and can be expensive and time consuming to develop and validate (Abegglen *et al.*, 2018; Songthammanuphap *et al.*, 2020). Therefore, due to the relatively conserved gene sequences of cytokines, as well as the ease and cost effectiveness of DNA sequencing, cytokine gene expression assays (GEAs) have been used as an alternative to antibody-based tests for several wildlife species to investigate and measure

cell-mediated immune (CMI) responses *in vitro* (Landolfi *et al.*, 2010; Higgitt *et al.*, 2017; Olivier *et al.*, 2017; Roos *et al.*, 2019). Cytokine GEAs measure components of CMI responses which are initiated during different stages of infection and allow for rapid, blood-based testing (Maas, Michel and Rutten, 2013).

Various candidate cytokine biomarkers such as *IFNγ*, *TNF*, and *TGFβ* have been investigated in Asian elephants (Landolfi *et al.*, 2010; Angkawanish *et al.*, 2013; Paudel *et al.*, 2016; Songthammanuphap *et al.*, 2020). A study done by Landolfi *et al.* (2010) demonstrated the potential use of a cytokine GEA to differentiate between suspect *M. tb*-infected and uninfected Asian elephants. Therefore, the development of cytokine GEAs in African elephants may help elucidate host immune responses and enable the development of potential diagnostic assays for this species.

As a first step in detecting CMI responses in African elephants, the aim of this study was to develop a GEA to measure cytokine expression (immune activation) in RNA extracted from mitogen stimulated whole blood and to determine which cytokines show promise as possible biomarkers of immune activation. The basis of a cytokine GEA relies on sequence-specific nucleotide primers which are designed using host mRNA transcripts. Since the sequences currently available for African elephants are predicted sequences, generated using computational analysis, the first objective of this study was to sequence full-length mRNA transcripts for selected host reference and target cytokine genes. The second objective was to use the confirmed mRNA transcripts to design sequence-specific primers and develop a real-time, reverse-transcription quantitative PCR (RT-qPCR) assay. The third objective was to characterize the temporal gene expression of these cytokines, to determine the optimal stimulation time at which to measure immune responses in this species. Finally, this assay was then used to identify candidate biomarkers of *in vitro* non-antigen specific immune activation in African elephants to advance the understanding of immune responses in this species.

2.2. Materials and Methods

2.2.1. Animals and sample collection

Whole blood samples for this study were collected from 28 free-ranging African bull elephants from Kruger National Park (KNP), South Africa. All sample collection was done opportunistically during routine immobilizations for management or veterinary procedures. Briefly, whole blood was collected from the auricular vein into BD Vacutainer® lithium heparin tubes (Becton Dickinson, Franklin Lakes, NJ, USA) and transported to the laboratory at room temperature within 4-6 hours of collection. Two elephants were classified as young adults (≥ 24 years), with 26 individuals classified as adult (≥ 25 years) at the time of sampling.

Ethical approval for the sample acquisition and testing of these animals was granted by the Stellenbosch University Animal Care and Use Committee (SU-ACU 2018-6308), and South African National Parks Animal Care and Use Committee (SANParks Research Agreement BUSP1511). Section 20 approval was granted by the South African Department of Agriculture, Land Reform and Rural Development (DALRRD) formerly the Department of Agriculture, Forestry, and Fisheries (DAFF Section 20: 12/11/1/7/6).

2.2.2. Whole blood stimulation

Whole blood stimulation was performed using nil (phosphate buffered saline (PBS)) and pokeweed mitogen (PWM) in-tube stimulations prepared as follows: one mL heparinized whole blood was transferred into a Vacutainer® tube (BD Biosciences) containing either 10 µl PBS (Thermo Fisher Scientific, Waltham, MA, USA) or 10 µl PWM (10 µg/ml final concentration in PBS) (Sigma-Aldrich, St. Louis, MO, USA). All whole blood stimulations were performed in duplicate; tubes were inverted several times and incubated at 37°C for 6 and 24 hours. For one component of the study, replicate nil and PWM stimulated whole blood from three individuals were incubated for 0, 4, 6, 8, 10, 12, 18 and 24 hours. Following incubation, blood was transferred to a 2 mL microcentrifuge tube and centrifuged at 2 000 x g for 15 minutes. The plasma fraction was harvested, and the remaining cell pellet resuspended in 1 mL RNALater® Solution (Ambion, Foster City, CA, USA). Both plasma and cell pellets, stabilized in RNALater®, were stored at -80°C.

2.2.3. RNA extraction and reverse transcription

The RiboPure™-Blood Kit (Ambion) was used to extract RNA from RNALater® stabilized cell pellets, according to the manufacturer's instructions, with the following modifications: RNA elution was performed in two steps. The first elution was performed using 30 µl elution buffer, incubated for 5 minutes at room temperature, and centrifuged for 30 seconds at 16 000 x g. The second elution was performed using 30 µl elution buffer, incubated for 3 minutes, and centrifuged for 1 minute at 16 000 x g to obtain a final volume of 60 µl total RNA. The quantity (ng) and A260/280 and A260/230 ratios of the extracted RNA were measured using the Nanodrop 1000 spectrophotometer (ThermoFisher Scientific), after which RNA was stored at -80°C prior to reverse transcription.

Reverse transcription was performed using QuantiTect® Reverse Transcription Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Each reaction contained genomic DNA (gDNA) Wipeout Buffer to ensure effective elimination of gDNA from initial RNA sample. An estimated total of 200 ng RNA was reverse transcribed to cDNA (total volume 20µl), after which cDNA was stored at -20°C prior to downstream analysis.

2.2.4. Primer design

A panel of potential immune biomarkers was selected for use in African elephants, based on previous studies in Asian elephants, spotted hyenas, African lions, and common warthogs (Landolfi *et al.*, 2010; Higgitt *et al.*, 2017; Olivier *et al.*, 2017; Roos *et al.*, 2019). Four reference genes were selected: actin-beta (*ACTB*), beta-2-microglobulin (*B2M*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (*YWHAZ*). A panel of eight immune mediator target genes were also selected: C-X-C motif chemokine ligands 9 (*CXCL9*) and 10 (*CXCL10*); interleukins 4 (*IL4*), 10 (*IL10*) and 12 (*IL12*); interferon gamma (*IFNγ*), transforming growth factor beta (*TGFβ*), and tumor necrosis factor (*TNF*).

Predicted African elephant mRNA transcripts derived by automated computational analysis using gene prediction methods were obtained from NCBI GenBank® genetic sequence database (Benson *et al.*, 2013) and the Ensembl Genome Browser (Cunningham *et al.*, 2019). In addition, Asian elephant mRNA transcripts for *ACTB, GAPDH, TGFβ, IFNγ, TNF, IL10, IL4,* and *IL12* were obtained from the NCBI GenBank® genetic sequence database. For *B2M, CXCL9, CXCL10,* and *YWHAZ,* Asian elephant mRNA transcripts were not available, and common warthog mRNA transcripts were obtained from the NCBI GenBank® genetic

sequence database (Benson *et al.*, 2013). Multiple sequence alignments of Asian elephant, common warthog, and predicted African elephant mRNA transcripts were performed using ClustalX version 2.1 (Larkin *et al.*, 2007). Based on sequence alignments, novel primers were designed and used in combination with previously designed Asian elephant primers (J. Landolfi, unpublished data). Where differences between African elephants and other species were observed, primers were adapted to include ambiguous nucleotides to increase chances of amplification. All oligonucleotide primers (Table 2.1) were synthesized by Integrated DNA Technologies (IDT Inc., Coralville, IA, USA).

Table 2.1: Primers used for the amplification and sequencing of African elephant mRNA transcripts.

Gene	Forward Primer (5'→3')	Reverse Primer (5'→3')	Product Size (bp)	Annealing Temperature (°C)	NCBI Genbank® Accession Number
ACTB	F_1: AAC GGC TCS GGY ATG TG	R_1: CAG AGC TTC TCC TTG ATG TCA CG	700	55	MT096344,
	F_2: AAG TTC GCC ATG GAC GAT GA	R_2: TCA TAG ATG GGC ACA GTG TG	509	55	MT096345
	F_3: GAC TAC CTC ATG AAG ATC CTC AC	R_3: GTG TAA CGC AAC TAA AGA CAG TC	596	55	
B2M	F_1: TTC ACC ATG CGT CTC TTC GT	R_1: TGA AAA CTC ACC CCA TTT CAC TAC	366	55	MT096346, MT096347, MT096348
CXCL9	F_1: ATC CCA CCA CTA TGA AGA AAA GTG	R_1: GTA AAG TGT TGT CTT ACG CAG TC	405	55	MT096349, MT096350
CXCL10	F_1: TCT CAG CAC CAT GGA CCA ACG T	R_1: TAC AGT TAT CAT GCT TCT CTC TGC	333	55	MT096351, MT096352
GAPDH	F_1: AAG ATY GTC AGC AAT GCY TCC	R_1: CCA GGA AAT GAG CTT GAC AAA	500	55	MT096353,
	F_2: GGA CTT CCT GGA GAT AGC AAA AT	R_2: CAT TGC TGA CAA TCT TGA GAG AGT	553	55	MT096354
	F_3: TAC ACT GAA GAC CAG GTT GTC TC	R_3: GAA ACT GTA GAG GAT GGG AGA TTC	306	55	
IFNγ	F_1: GAT CAA CTT TAC ACA GGA GCT ACT	R_1: TGA CCA TTA TTC TGA TGC TCT CC	567	55	MT096355, MT096356
IL4	F_1: ATG GGT CTC ACC TAC CAG CTG	R_1: CAC TTG GAG TAT TTC TCC TTC ATG ATC	400	58	MT096357
IL10	F_1: TCA ACC TAT GTA TAA AAG GGG GAC	R_1: GTC TAG TAG AGT CGC CAT GTT G	675	55	MT096358, MT096359

bp – base pairs

Table 2.1 (Continued)

Gene	Forward Primer (5'→3')	Reverse Primer (5'→3')	Product Size (bp)	Annealing Temperature (°C)	NCBI Genbank® Accession Number
IL12	F_1: CAG CCA CCG CCC TCA C	R_1: TGT GGC ACA GTC TCA CTG TTG A	500	55	MT096360,
	F_2: GCA CTT CTG AAG AGA TTG ACC ATG	R_2: AGA ATT ACG GTG CCA GCT TAA GTA	562	55	MT096361
TGFβ	F_1: CGC GTG CTA ATG GTG GAA A	R_1: GTG TCC AGG CTC CAR ATG TAG G	600	55	MT096362,
	F_2: GTG GAA ATC AAA GGG CTG AAT AAC	R_2: TCC TCT CTC CAC CTT TAA TGG G	600	55	MT096363
TNF	F_1: CTC TCC AAA GGA CAC CAT GAG C	R_1: ATG GGC ATC CAT TCC CCC TCA	742	55	MT096364, MT096365
YWHAZ	F_1: AAC ATC CAG TCA TGG ATA AAA A	R_1: CTA CTG TGT AAA TTT CAG AAT	796	55	MT096366, MT096367

bp – base pairs

2.2.5. PCR amplification and mRNA sequence confirmation

The PCR reactions to amplify mRNA transcripts were performed using 1 µl of cDNA in a 25 µl PCR reaction containing 12.5 µl of OneTaq® Hot Start 2X Master Mix with Standard Buffer (New England Biolabs Inc, Ipswich, MA, USA), 0.5 µl of each primer (10 µM) and 10.5 µl nuclease-free water. The PCR cycling conditions, using a Veriti® Thermal Cycler (Applied Biosystems, Foster City, CA, USA), were as follows: initialization at 94°C for 2 minutes, followed by 45 cycles of denaturation at 94°C for 30 seconds, annealing and elongation at 55°C for 30 seconds (58°C for *IL4*; Table 2.1), and extension at 68°C for 1 minute. Final extension was done at 68°C for 10 minutes. Negative (no template) controls were included in all PCR reactions. To confirm that cDNA was amplified and not gDNA carried over from the RNA extraction, PCR was performed using the following templates: extracted RNA only; extracted RNA treated with gDNA Wipeout Buffer (without reverse transcription); and extracted RNA treated with gDNA Wipeout Buffer and reverse transcribed to cDNA, as described in section 2.2.3.

Amplicon sizes were estimated following electrophoresis using a 1.5% agarose gel (Lonza Group, Basel, Switzerland). For each cytokine, sequences from two randomly selected elephants were determined and used for further analyses. Where sequences could not be confirmed in the initial two elephants, cDNA from a third animal was used to obtain sequences for some cytokines.

The PCR products from the selected African elephants were sent for post PCR clean-up and sequencing at the Stellenbosch University Central Analytical Facility (CAF; Stellenbosch, South Africa) using the ABI 3730XL 96-capillary DNA Analyzer (Applied Biosystems), according to the manufacturer's guidelines. Sequences were edited and aligned using the Sequencher® version 5.1 DNA sequence analysis software (Gene Codes Corporation, Ann Arbor, MI, USA; http://www.genecodes.com) as well as the Geneious® 6.0.6 software (Biomatters Ltd., Auckland, New Zealand; http://www.genious.com). Sequences were then authenticated using the NCBI Basic Local Alignment Search Tool (BLAST) software program (Altschuk *et al.*, 1990). Newly generated mRNA transcripts derived from this study were deposited in NCBI GenBank® and accession numbers listed in Table 2.1.

2.2.6. qPCR design

Novel qPCR primers were designed using the confirmed African elephant mRNA transcript sequences. Primers for four reference genes: *ACTB, B2M, GAPDH* and *YWHAZ*; and eight target genes: *CXCL9, CXCL10, IFNγ, IL4, IL10, IL12, TGFβ* and *TNF* were synthesized by Integrated DNA Technologies (IDT Inc.) (Table 2.2).

To determine the suitability of qPCR primers, cDNA from five randomly selected African elephants was amplified using both conventional PCR and qPCR. Conventional PCR was performed as described in section 2.2.5, with the following PCR cycling conditions: initialization at 94°C for 2 minutes, followed by 45 cycles of denaturation at 94°C for 30 seconds, annealing and elongation at 60°C for 30 seconds and extension at 68°C for 1 minute. Finally, extension was performed at 68°C for 10 minutes. Negative (no template) controls were included in all PCR reactions. Amplicon sizes were estimated following electrophoresis using a 1.5% agarose gel, and PCR products were sent for post PCR clean-up and sequencing at CAF. Sequences were edited and aligned using the Sequencher 5.1 software and authenticated using the NCBI BLAST software program.

To confirm amplification using qPCR, cDNA from the two African elephants, used for PCR amplification and sequencing, was run on the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). Each gene target was amplified separately in a 10 µl PCR reaction containing 1 µl of cDNA, 5 µl iTAQ™ Universal SYBR® Green Supermix (Bio-Rad), 0.4 µl of each forward and reverse primer (final concentration of 400 nM), and nuclease-free water. The qPCR cycling conditions were as follows: polymerase activation at 95°C for 30 seconds, followed by 40 cycles of denaturation at 95°C for 5 seconds, and annealing and elongation at 60°C for 30 seconds. Melt-curve analysis was performed over a 65–95°C range with increased increments of 0.5°C every 5 seconds. The melt curve of each product was characterized and used to confirm the specificity of subsequent qPCRs.

2.2.7. Selection of reference and target genes

The amplification efficiencies of both reference and target genes were determined using five-fold serial dilutions which were prepared as follows: RNA was extracted from PWM stimulated whole blood from three African elephants and an estimated total of 1000 ng RNA was reverse transcribed for each elephant as described in section 2.6. The cDNA was then pooled, and a serial dilution prepared (1:5, 1:25, 1:125, 1:625, 1:3125, 1:15625), with an estimated range of 1000 to 0.064 ng. Each dilution point was run in triplicate using the qPCR cycling conditions described above, and amplification efficiencies were determined as previously described (Livak and Schmittgen, 2001). Reference and target genes with RT-qPCR efficiencies of 90-110% were selected for further analysis. To validate the use of the relative quantification method described in (Livak and Schmittgen, 2001), the amplification efficiencies of all reference gene RT-qPCRs were compared to those of the target genes to evaluate compatibility. The final subset of target genes was chosen as those that had amplification RT-qPCR efficiencies within the recommended range, and were compatible with an efficient, stable reference gene.

Relative expression stability for each reference gene was determined using RNA extracted from nil and PWM stimulated whole blood of three African elephants. A total of 200 ng of RNA was reverse transcribed into 20 µl cDNA, and 1 µl cDNA was amplified using the RT-qPCR as described in section 2.2.3. The amplification stability of each reference gene was then determined using the geNorm applet in Microsoft Excel (Vandesompele *et al.*, 2002) and the NormFinder Excel Add-In (Andersen, Jensen and Ørntoft, 2004). The coefficient of variance was calculated using triplicate reactions to determine the intra-assay variability (IAV).

2.2.8. Proof of concept for using GEA to detect immune activation

To determine the utility of the GEA to measure cytokine expression in mitogen stimulated samples (as an indication of immune activation), the relative abundance of the target genes was measured over a 24-hour incubation period in three African elephants. Cytokine expression was measured at time points 0, 4, 6, 8, 10, 12, 18 and 24 hours, to determine the optimal stimulation time. Following initial temporal results, cytokine expression was measured for 16 African elephants, with nil and PWM stimulation samples at both 6 and 24 hours. For all samples, RNA was extracted, 200 ng of RNA reverse transcribed to 20 µl cDNA, where after RT-qPCR reactions were performed using the conditions described in section 2.2.6.

2.2.9. Data analysis

The relative fold change ($^{2-\Delta\Delta Cq}$) was used to measure up-regulation of the target genes in response to mitogen stimulation (Livak and Schmittgen, 2001). Relative gene expression of the target genes was normalized to a selected reference gene that is continuously expressed. For each stimulation (nil and mitogen), the mean reference gene Cq value was subtracted from the mean target gene Cq to determine the relative abundance of the target gene mRNA (ΔCq). The ΔCq derived from the nil tube was then subtracted from that of the mitogen tube (ΔΔCq). Thereafter, the relative fold change in abundance of the target transcript ($^{2-\Delta\Delta Cq}$) was measured and calculated as previously described (Livak and Schmittgen, 2001). Up-regulation was classified as a minimum fold change ($^{2-\Delta\Delta Cq}$) of > 1 while fold change <1 was classified as down-regulation (Radonic *et al.*, 2004; Schmittgen and Livak, 2008). A paired Student's t-test was used to compare mitogen expression at 6 and 24 hours. A p-value < 0.05 was considered statistically significant. Statistical analyses were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, La Jolla, CA, USA; www.graphpad.com). The Spearman Rank correlation coefficient was calculated using Excel software (Microsoft Excel version 2011, Redmond, WA, USA).

2.3. Results

Blood samples opportunistically obtained from 28 African elephants were used in this study. The extracted RNA yield from the 28 African elephant whole blood samples ranged from 92 to 4076 ng (median: 1258 ng). The A260/280 ratio ranged from 1.7 to 2.1 (median: 2.0) and the A260/230 ratio ranged from 0.1 to 8.6 (median: 2.1). Where A280/A260 and A260/230 ratios were outside the recommended range, samples were still used for further analysis due to the limited availability of samples for this species. Using conventional PCR, full coding sequences, determined using the NCBI BLAST CDS predictor, were obtained for *CXCL9, CXCL10, IFNγ, IL4, IL12,* and *YWHAZ*; and partial coding sequences were obtained for *ACTB, B2M, GAPDH,* and *TNF*. One full and one partial coding sequence for both *IL10* and *TGFβ* were obtained (Table 2.1). High nucleotide sequence identity was observed when mRNA sequences generated from African elephant whole blood, during this study, were compared to published Asian elephant (96.6-99.8%) and common warthog sequences (72-97.6%).

The suitability of newly designed qPCR primers (Table 2.2), designed using African elephant mRNA transcripts (NCBI GenBank® Accession numbers MT096344-M096367; Table 2.1), was confirmed for all four reference genes (*ACTB, B2M, GAPDH*, and *YWHAZ*) and five out of the eight target genes (*IL4, IL10, IL12, TGFβ*, and *TNF*), following the successful amplification and sequence confirmation of mRNA transcripts from mitogen stimulated African elephant whole blood samples. Amplification of *CXCL9, CXCL10* and *IFNγ* using RT-qPCR was unsuccessful and these genes were subsequently excluded from further analysis. Using the characteristic melt curve peaks at each dilution point to confirm the amplification of each gene, the RT-qPCR amplification efficiencies were calculated for four reference (*ACTB, B2M, GAPDH,* and *YWHAZ*) and four target (*IL4, IL10, TGFβ,* and *TNF*) genes, and fell within the recommended range of 90-110% (Table 2.2, Appendix Figures 1 and 2) (Livak & Schmittgen, 2001; Pfaffl, 2001) while the amplification efficiency of *IL12* was above 110% and therefore excluded from downstream analysis. The intra-assay variability for all genes was below 5%.

Using nil and PWM stimulated whole blood samples, *B2M* and *GAPDH* were identified as the most stably expressed reference genes (Appendix Figure 3). When comparing the reference gene amplification efficiencies with that of the target genes to assess compatibility, *GAPDH* was most suitable within relative quantification methods, along with *IL10, TGFβ*, and *TNF*,

and was chosen as the optimal reference gene. Subsequently, *IL4* was excluded from downstream analysis as it was not compatible with any of the reference genes.

The mitogen-induced expression of *IL10, TNF,* and *TGFβ* was measured over time in samples from three African elephants. Both *IL10* and *TNF* showed up-regulation over time (Figure 2.1A and B). For *IL10*, the highest median fold change was observed at 0, 6, and 24 hours of stimulation. For *TNF*, the highest median fold change was observed at 0, 6, and 18 hours of stimulation. Expression of *TGFβ* was considered down-regulated as median fold change was < 1 for all time points, except at 0 hours (Figure 2.1C).

Table 2.2: African elephant qPCR primer sequences and assay parameters determined during the development of the gene expression assay, including the derivative melt curve peak (DM), amplification efficiency (E), R^2 and the intra-assay variability (IAV).

Gene	**Forward Primer (5' → 3')**	**Reverse Primer (5' → 3')**	**Product size (bp)**	**DM peak (°C)**	**E (%)**	**R^2**	**IAV (%)**
ACTB	ACCCTGTGTTGCTGACTGAG	CAGAGGCATACAGGGACAGC	135	84	109.1	0.999	1.4
B2M	GCTTGCTCCAAAGGTTCAGG	GGTGGATGAAACCCAGACACA	93	78	102.6	0.998	0.7
GAPDH	ACGTGTCAGTGGTGGATCTG	GCCTGCTTCACCACCTTCTT	79	78.5	104.7	0.999	0.9
YWHAZ	ACTACCGTTACTTGGCTGAGG	CTGATAGGATGCGTTGGTTGC	127	79	96.4	0.977	0.9
IL4	CAGGTCTCTAAACGCCACGA	CCAGGTTTGTCATGCTGCTG	79	78	99.6	0.995	1.6
IL10	CGGTGGAGCAAGTGAAGGAA	TGTCAAACTCACTCATGGCC	75	77.5	96.3	0.998	1.9
IL12	GGCCAGACAAACCCTAGAGT	CCAGGCAATTCTCATTCGCG	131	79	143.6	0.993	2.0
TGFb	TCCACCGAGAAGAACTGCTG	GCTCCAGATGTAAGGGCAGG	132	85	99.1	0.997	1.1
TNF	CCTGTAGCACACGTTGTAGC	CATCGGAAGGCACCATCAGC	127	82	101.7	0.999	1.0

bp – base pairs

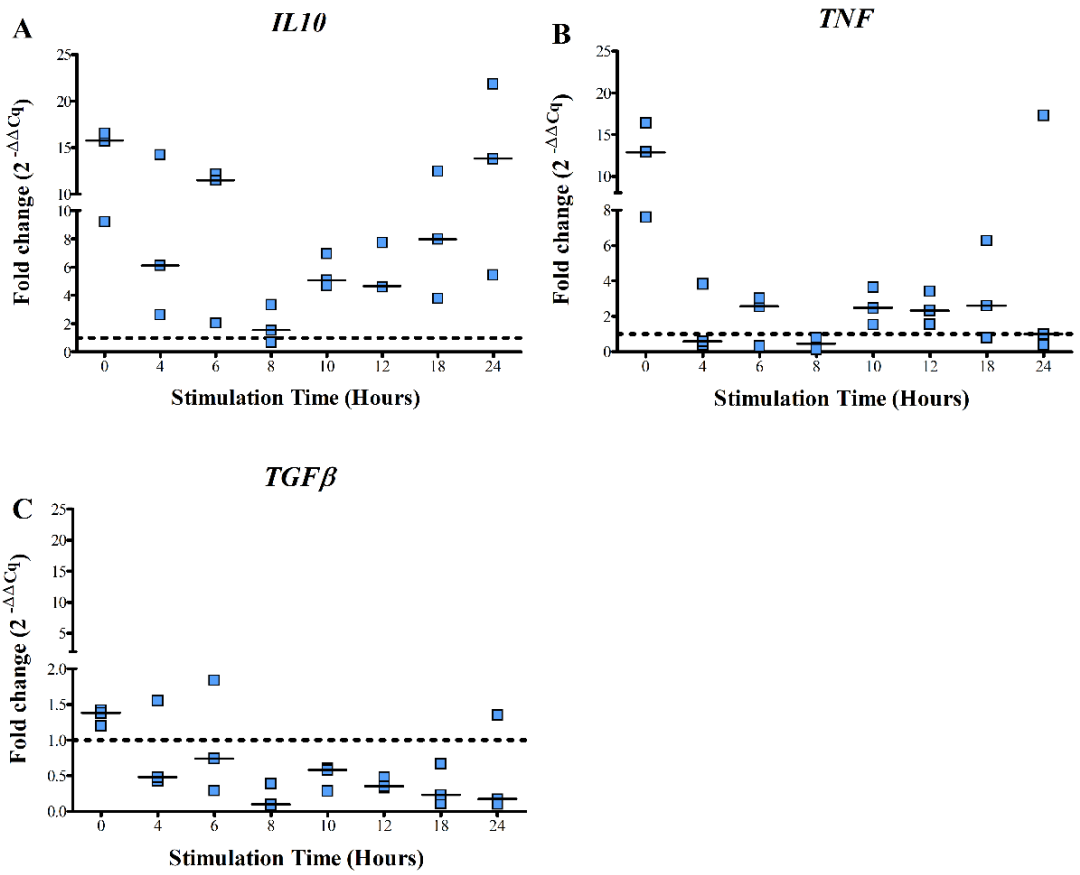


Figure 2.1: Gene expression of *IL10* (A), *TNF* (B), and *TGFβ* (C) measured in three African elephants over 24 hours following pokeweed mitogen (PWM) stimulation. The median expression at each time point is indicated by a solid horizontal line. The dotted line on the y-axis indicates the minimum fold change ($2^{-\Delta\Delta Cq} > 1$) for expression to be considered up-regulated.

Samples from 16 African elephants were available for 6- and 24-hour stimulation time points. The median fold change for *IL10* was measured as 16.9 and 18.5, at 6 and 24 hours, respectively (Figure 2.2A), indicating up-regulation at both time points. No significant difference was seen between the two time points. For *TNF*, median fold change was 2.1 and 8.1, at 6 and 24 hours, respectively (Figure 2.2B). However, at the 6-hour time point, 3 of the 16 samples had a fold change for *TNF* that was < 1, with median fold change significantly higher at 24 hours ($p = 0.0035$). For *TGFβ*, median fold change was 0.5 and 1, at 6 and 24 hours, respectively (Figure 2.2C), indicating down-regulation of this gene at the 6-hour time point. Mitogen stimulation thus did not result in the up-regulation of *TGFβ*, and there was no significant difference between the time points.

The distribution of cytokine responses shown in Figure 2A and 2B appeared to have some separation into high and low expressors, especially for *IL10* and *TNF* at 24 hours stimulation. When individual elephant values were compared, of the 8 elephants that had *IL10* fold changes greater than the median at 24 hours, only 4 of these animals also had TNF fold changes greater than the median. The Spearman rank correlation coefficient (Rs) was -0.0147, which indicates essentially no correlation between *IL10* and *TNF* fold change values for individual elephants. A similar coefficient (Rs = 0.0147) was calculated for the association between *TNF* and *TGFβ*, although there appeared to be a weak correlation between *IL10* and *TGFβ* expression (Rs = 0.324).

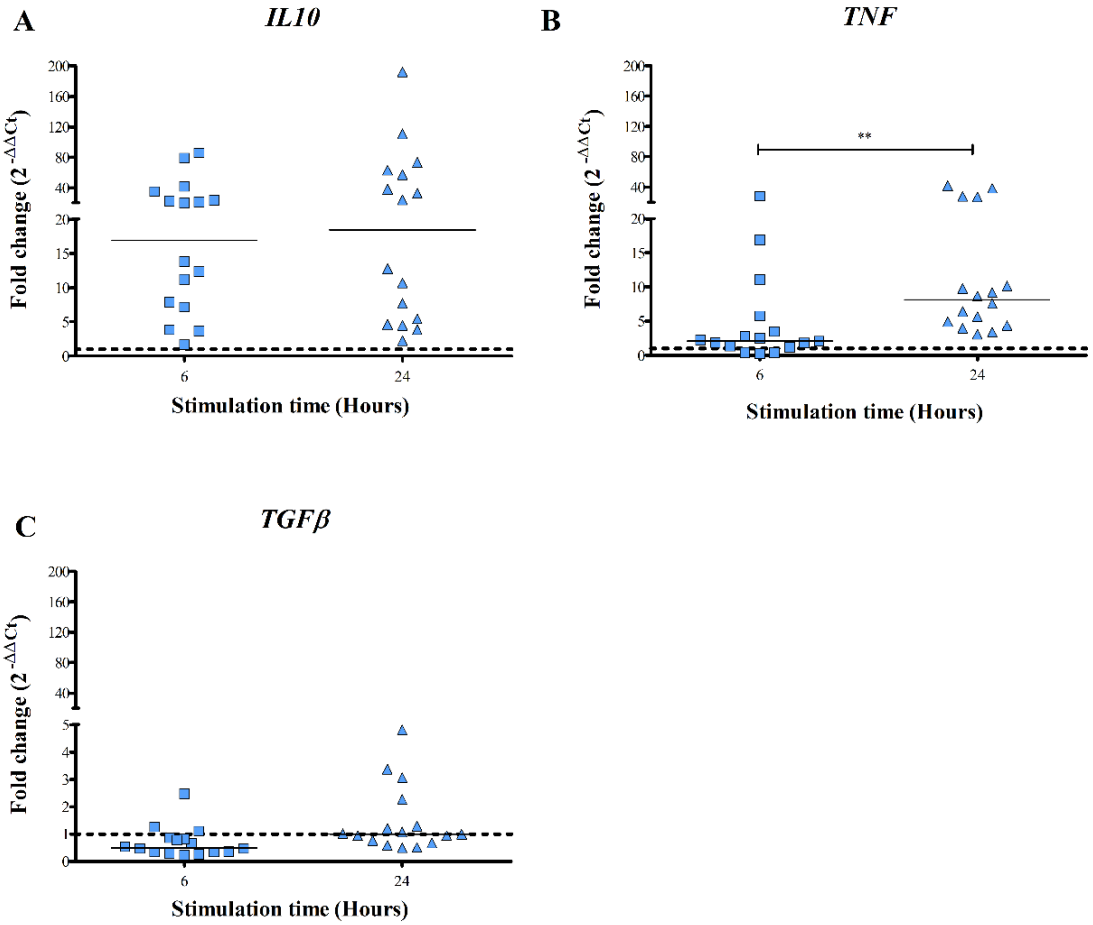


Figure 2.2: Gene expression of *IL10* (A), *TNF* (B) and *TGFβ* (C) measured in 16 African elephants measured at 6 and 24 hours, following pokeweed mitogen (PWM) stimulation. The median fold change for each group is indicated by a solid horizontal bar. The dotted line on the y-axis indicates the minimum fold change ($2^{-\Delta\Delta Cq}$ >1) for expression to be considered up-regulated. ** $p < 0.01$.

2.4. Discussion

Cell-mediated immune responses in elephants have not been well characterized. Since immunoassays are often based on detecting and measuring changes in cytokines, and can be the basis for diagnostic tests, the aim of this study was to identify biomarkers and develop a cytokine GEA for use with whole blood samples in African elephants. The findings presented included the first report of full coding sequences for the reference gene *YWHAZ*, and immune mediator target genes (*CXCL9, CXCL10, IFNγ, IL4* and *IL12*), as well as partial coding sequences for *ACTB, B2M, GAPDH* and *TNF*, and one full and one partial coding sequence for both *IL10* and *TGFβ*. These confirmed African elephant sequences provided the basis for developing real-time RT-qPCR assays. Future studies should consider sequencing additional animals to confirm these sequences in a larger cohort of African elephants.

Using the newly confirmed coding sequences, an African elephant cytokine GEA was developed. Of the reference and target genes evaluated, *GAPDH* was identified as the most stable and efficiently expressed reference gene and was compatible with three cytokine target genes (*IL10, TGFβ,* and *TNF*). Similarly, in a real-time RT-PCR, validated for Asian elephants, *GAPDH* was also identified as the best reference gene and cytokines *IL10, TGFβ,* and *TNF* could be detected in RNA extracted from unstimulated blood samples, albeit at low levels (Landolfi *et al.*, 2010). Despite low level amplification of *IL4* and *IL12* in Asian elephants (Landolfi *et al.*, 2010), and the use of African elephant specific qPCR primers, we were unable to develop an assay for these markers. This was due to the lack of compatibility between *GAPDH* and *IL4*, out of range RT-qPCR amplification efficiency of *IL12*, and the lack of consistent amplification of *CXCL9*, *CXCL10*, and *IFNγ*, which precluded their use in the cytokine GEA. Attempts at PCR temperature optimization was unsuccessful using the current primers designed for these genes, and further studies are needed to optimize qPCR primers and reaction conditions for these targets in African elephants.

Mitogen stimulation of whole blood is expected to result in general immune activation, detected by changes in relative abundance of cytokines measured in GEAs, which can be used as a proof of concept (positive control) for antigen-specific cytokine GEAs. A time point series of stimulated whole blood samples was used to identify optimal incubation times for detection of the three target cytokine genes. The median expression of *IL10* showed a cyclic pattern over the 24-hour period, with up-regulation highest at 0, 6 and 24 hours, while the expression patterns of *TNF* and *TGFβ* were highest at 0 and 6 hours, followed by a decline in expression

over time, and TGFβ showing slight down-regulation. Although there was variation in the patterns of expression between the three African elephants tested, the median up-regulation of all three cytokines at 0 hours suggests that activation of circulating immune cells had occurred prior to experimental stimulation. A possible explanation might be that immune activation occurred within the 15 minutes that the stimulated whole blood was centrifuged for separation before they were stored at -20°C. Since the elephants had been herded by a helicopter during induction of immobilization, this may have resulted in release of catecholamines, which have been shown to induce both pro-inflammatory and anti-inflammatory responses in macrophages (Barnes, Carson and Nair, 2015; Shaw *et al.*, 2018). Furthermore, glucocorticoids (GCs) produced as part of the physiological stress response may have resulted in the down-regulation of inflammatory mediators, including TNF, and decreased the stability of the cytokine mRNA, effects which are typically seen several hours after GCs are produced (Reichardt *et al.*, 2001; Anderson *et al.*, 2004). These two factors may have resulted in the biphasic response observed during the temporal component of our study. However, we did not test for the presence of catecholomines or GCs in our samples and no definitive conclusions can be drawn regarding their presence. Therefore, further studies using African elephants trained for blood collection should be performed to determine the impact of capture and immobilization on cytokine gene expression.

In this study, GEAs for cytokines *TNF, TGFβ,* and *IL10* were developed to further facilitate research on immune responses in African elephants. Tumor necrosis factor forms part of the acute phase response and is produced by activated macrophages/monocytes, lymphocytes, and natural killer (NK) cells (Wang, Czura and Tracey, 2003). This cytokine plays an important role in mediating cellular responses, including enhanced natural killing cell function, induction of apoptosis in mature T-cells, and neutrophil activation (Rath and Aggarwal, 1999; Wang, Czura and Tracey, 2003; Abbas, Lichtman and Pillai, 2016). A study by Landolfi *et al.* (2010), using RNA extracted from unstimulated blood samples, showed that Asian elephants, suspected to be infected with *M. tb*, had significantly higher expression of *TNF* than uninfected elephants. In this study, *TNF* was up-regulated in mitogen stimulated whole blood of African elephants at both 6 and 24 hours, with significantly higher expression at 24 hours. These findings support further investigation of *TNF* to evaluate the diagnostic potential of this cytokine in infections and inflammation in African elephants as seen in Asian elephants.

Gene expression of two anti-inflammatory cytokines (*IL10* and *TGFβ*) was also examined in this study. Interleukin 10 is an important cytokine that regulates immune responses to

pathogens, limits immunopathology and plays a role in wound healing (Sabat *et al.*, 2010; Saraiva, Vieira and O'Garra, 2019). Previous studies have shown that increased expression of *IL10* promotes disease progression during *M. tb* infection (Beamer *et al.*, 2008), while *IL10* knockout mice showed increased immune-related pathology (Higgins *et al.*, 2009). In this study, *IL10* expression was up-regulated at 6 and 24 hours, following mitogen stimulation of African elephant whole blood. The relatively high level of up-regulation may be related to the nonspecific expression of *IL10* by a wide range of cell types (including almost all leukocytes) (Sabat *et al.*, 2010; Saraiva, Vieira and O'Garra, 2019). Mitogen stimulation results in the general activation of circulating immune cells, resulting in the increased expression of *IL10* in examined peripheral blood samples. Based on its crucial role as a mediator of inflammation in a variety of diseases, and detection of up-regulation in this study, *IL10* should be included in further studies on immune responses in African elephants.

Transforming growth factor beta also has anti-inflammatory properties and is involved in regulatory activities including T-cell suppression and macrophage activation (Toossi and Ellner, 1998; Travis and Sheppard, 2014). However, expression of *TGFβ* may also promote inflammation under certain conditions (Sanjabi *et al.*, 2009). In contrast to *TNF* and *IL10*, slight down-regulation of *TGFβ* expression was observed at both 6 and 24 hours in stimulated blood samples from African elephants. Down-regulation of *TGFβ* was also previously observed in unstimulated whole blood samples of TB-seropositive Asian elephants (Landolfi *et al.*, 2010). In addition, the production of *TGFβ* has been shown to have inhibitory effects on B lymphocytes (Kehrl *et al.*, 1986; Sanjabi, Oh and Li, 2017). However, previous studies in Asian elephants with TB have shown a robust humoral response in infected individuals (Greenwald *et al.*, 2009). Possibly, such findings could be due to down-regulation of *TGFβ* expression during immune responses in elephants. More research is needed to determine the role that this cytokine plays in elephants. Although the low expression of this gene limits its use as a single diagnostic marker in African elephants, the observed slight down-regulation could be useful in a biomarker panel, as seen in humans (Dhanasekaran *et al.*, 2013; Han *et al.*, 2018).

When cytokine expression was compared among individual elephants, there appeared to be high and low expressors, especially of *IL10* and *TNF*, at 24 hours. However, there was poor correlation between fold change values of these cytokines by the same individual animal. Similarly, this was also true for the correlation between *TNF* and *TGFβ*, which may be expected since TNF is a pro-inflammatory cytokine and *IL10* and *TGFβ* are anti-inflammatory (Wang, Czura and Tracey, 2003; Sanjabi et al., 2009). Therefore, it seems reasonable that a greater

correlation was observed in values for the two anti-inflammatory cytokines *IL10* and *TGFβ*, however the role of these cytokines in elephant immune responses requires further investigation.

Limitations in this study were the small number of samples used for cytokine gene sequencing (n=2) and temporal patterns (n=3) of cytokine expression in response to mitogen stimulation. In addition, all elephants were free-ranging individuals, requiring capture and chemical immobilization, which may have resulted in acute physiological stress responses impacting immune stimulation. Therefore, further studies with a larger cohort of African elephants are needed to confirm the observations reported. In addition, a comparison of mitogen responses using trained African elephants to evaluate the influence of capture on cytokine expression would facilitate interpretation. Importantly, additional cytokine genes should be investigated to provide a broader set of tools for studying immune responses in this species.

2.5. Conclusion

To our knowledge, this is the first study investigating cytokine biomarkers in African elephants using real-time RT-qPCR. The cytokine sequences used in this study are the first confirmed mRNA transcripts for this species. The up-regulation of *IL10* and *TNF*, as well as the slight down-regulation of *TGFβ* demonstrate the possible use of these biomarkers to measure immune responses to inflammatory and infectious diseases. The knowledge generated will provide a foundation for future assay development.

Chapter 3: Evaluation of mycobacterial antigen stimulated responses in African elephant (*Loxodonta africana*) whole blood using cytokine gene expression assays

In this chapter, African elephant (n = 11) whole blood samples were stimulated with antigens ESAT6/CFP10, *M. avium* purified protein derivative (PPDa) and *M. bovis* purified protein derivative (PPDb) for 6 hours. Following antigen stimulation, RT-qPCRs for target genes *IL10, TGFβ,* and *TNF* were performed and normalized to reference gene *GAPDH,* to determine cytokine gene abundance which was used to assess their potential use as biomarkers for TB in elephants.

3.1. Introduction

Tuberculosis (TB) in elephants is most commonly caused by infection with the intracellular pathogen *Mycobacterium tuberculosis* (*M. tb*), a member of the *Mycobacterium tuberculosis* complex (MTBC) (Fowler, 2008), while a small number of cases caused by *Mycobacterium bovis* (*M. bovis*) have been reported (Lyashchenko *et al.*, 2006; Goosen *et al.*, 2020a). Tuberculosis is a chronic infection, with antemortem signs often absent until the disease has progressed to advanced stages (Mikota and Maslow, 2011; Paudel and Toshio, 2016). Since elephants with *M. tb* infection were documented in the USA in the 1990's, many other cases have been reported globally (Mikota *et al.*, 2001; Paudel and Sreevatsan, 2020). While most of these were identified in captive Asian elephants (*Elephas maximus*), there have been several cases of TB documented in free-ranging Asian elephants in Sri Lanka and India (Perera *et al.*, 2014; Chandranaik *et al.*, 2017; Zachariah *et al.*, 2017). To date, much of the disease research has focused on infection in Asian elephants, however, African elephants (*Loxodonta africana*) are also susceptible and impacted by this disease. The first fatal cases of TB in free-ranging African elephants were documented in Tsavo East National Park, Kenya (Obanda *et al.*, 2013) and the Kruger National Park (KNP) in South Africa (Miller *et al.*, 2019). Two cases of *M. tb* infection were also diagnosed in African elephants at the South African National Biodiversity Institute National Zoological Gardens (SANBI-NZG) (unpublished data). Since TB in elephants is considered an anthropozoonotic disease, elephant interactions with humans have raised concerns with regards to impacts on conservation (Zachariah *et al.*, 2017; Lainé, 2018).

African elephants are listed as vulnerable on the International Union for Conservation of Nature's (IUCN) Red List of Threatened Species, due to the ongoing threat of poaching and illegal hunting (Blanc, 2008). Additional threats facing African elephants include habitat

fragmentation and animal-human conflict (Fowler, 2008; Sach *et al.*, 2019; de Sales, Anastácio and Pereira, 2020), however the threat of disease should not be underestimated. It is thus important to understand the effects of TB in this species.

There are currently only a limited number of diagnostic tests that have been validated for the detection of TB in elephants. Current antemortem tests include serological assays and mycobacterial culture (Fowler, 2008; Greenwald *et al.*, 2009; Lyashchenko *et al.*, 2012). Serological assays can provide important information regarding previous exposure to MTBC organisms, however, seropositivity does not equate with current infection. There is also a paucity of data regarding the seroprevalence of MTBC in free-ranging African elephants, with the first study reported recently by Kerr *et al.* (2019). This study was performed retrospectively on serum samples from the KNP population, and found an estimated prevalence of 6%, based on results from the Dual Path Platform (DPP®) Vet TB Assay for Elephants (DPP, Chembio Diagnostic Systems, Inc., Medford, NY, USA), and 9% based on the Elephant TB STAT-PAK Assay (Chembio). Several studies have used interferon-γ release assays (IGRAs) in Asian elephants, however their use in African elephant populations has not been investigated (Angkawanish *et al.*, 2013; Paudel *et al.*, 2016; Songthammanuphap *et al.*, 2020). Mycobacterial culture of respiratory samples, including trunk wash (TW) and bronchoalveolar lavage (BAL) samples, is currently viewed as the gold standard for confirming MTBC infection, however respiratory sample collection and mycobacterial culture are expensive, laborious and samples, in particular TW, are often contaminated, negating results (Hermes *et al.*, 2018). Therefore, rapid, and accurate antemortem diagnostic tests are needed.

Since antigen-specific cell-mediated immune (CMI) responses are detectable following *M. tb* infection (Abegglen *et al.*, 2018), cytokine gene expression assays (GEAs) have been used to diagnose MTBC infection in elephants and various other species (Landolfi *et al.*, 2010; Clarke *et al.*, 2017; Higgitt *et al.*, 2017; Olivier *et al.*, 2017). A study done by Landolfi *et al.* (2010) reported significantly higher levels of tumor necrosis factor (*TNF*) and lower levels of transforming growth factor beta (*TGFβ*) gene expression in unstimulated peripheral whole blood samples from TB seropositive Asian elephants using real-time, reverse-transcription quantitative PCR (RT-qPCR). Since GEAs make use of species-specific primers, which are designed to target a cytokine gene of interest, in combination with commercially available qPCR master mixes, application may be more feasible and cost-effective than antibody-based tests in species where antibodies are not commercially available (Landolfi *et al.*, 2014; Abegglen *et al.*, 2018). Therefore, as a proof of concept, the aim of this study was to measure

levels of gene expression following 6-hour incubation with negative control (nil), MTBC antigens (PPDa, PPDb, QFT-TB2) and mitogen (PWM) stimulation of heparinized whole blood collected from African elephants. Stimulants were selected to provide an indication of circulating/baseline cytokine levels (nil); response to environmental mycobacteria (PPDa); response to *M. bovis* antigens (PPDb); response to TB-specific antigens (QFT-TB2), and general immune activation (PWM). Additionally, the differential PPD response (PPDb-PPDa) was also calculated as an indication of a TB-specific response. Previously designed and optimized RT-qPCRs for target genes interleukin 10 (*IL10*)*, TGFβ,* and *TNF*, normalized to reference gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (de Waal *et al*., 2021; Chapter 2), were used to assess their potential utility as biomarkers for TB in elephants. During the development of the RT-qPCRs (de Waal *et al*., 2021; Chapter 2), 24-hour whole blood stimulation was determined as optimal in African elephants. However, as heparinized whole blood was collected prior to development of RT-qPCRs, only samples stimulated with the panel of TB antigens for 6 hours were available for this part of the study.

3.2. Materials and Methods

3.2.1. Animals and sample collection

Samples for this study were opportunistically collected from 11 free-ranging African bull elephants from Kruger National Park (KNP) in South Africa, which is endemic for *M. bovis*, during routine immobilizations for management or veterinary procedures. Whole blood was collected into BD Vacutainer® lithium heparin tubes (Becton Dickinson, Franklin Lakes, NJ, USA) and kept at room temperature until transported to the lab within 4-6 hours of collection. In addition, serum (BD Vacutainer®) and respiratory samples (BAL and TW) were also collected. At the time of sampling, four African elephants were classified as young adults (≤ 24 years), while seven were classified as adults (≥ 25 years).

Ethical approval for the sample acquisition and testing of these animals was granted by the Stellenbosch University Animal Care and Use Committee (SU-ACU 2018-6308), and South African National Parks Animal Care and Use Committee (SANParks Research Agreement BUSP1511). Section 20 approval was granted by the South African Department of Agriculture, Land Reform and Rural Development (DALRRD) formerly the Department of Agriculture, Forestry, and Fisheries (DAFF Section 20: 12/11/1/7/6).

3.2.2. Classification of disease status

The MTBC infection status of each African elephant was evaluated as follows. Serum samples were classified as serologically negative or positive using the DPP® Vet TB Assay for Elephants (Chembio) as previously described (Kerr *et al.*, 2019). This assay uses MTBC antigens, MPB83, early secretory antigenic target 6 kDa (ESAT6), and culture filtrate protein 10 kDa (CFP10), to detect the presence of antigen-specific antibodies in sera, which provides an indirect diagnosis of TB in elephants (Greenwald *et al.*, 2009).

Respiratory samples (TW and BAL) were collected for mycobacterial culture using the BD BACTEC™ MGIT™ 960 automated Mycobacterial Detection System (Becton Dickinson Biosciences). Trunk wash was performed by infusing sterile saline through a polypropylene tube placed into the trunk of the immobilized elephant, the trunk was then raised and lowered repeatedly, after which fluid was aspirated into a sterile container. After transport to the lab, samples were concentrated by centrifugation, and stored in 4 mL aliquots at -80°C prior to analysis (Goosen *et al.*, 2020a). Bronchoalveolar lavage samples were collected using a flexible endoscope as previously described (Goosen *et al.*, 2020a) and fluid processed similar to the

trunk wash samples prior to analysis. Concentrated BAL or TW samples (4 mL) were then decontaminated and processed for mycobacterial culture as previously described by Goosen *et al.* (2014). Each elephant was then classified as either MTBC infected or uninfected based on the presence or absence of mycobacterial growth and serological results.

3.2.3. Whole blood stimulation

For each African elephant, heparinized whole blood was stimulated using tubes prepared as follows: 25 µl phosphate buffered saline (PBS; nil), PPDa (Onderstepoort Biological Products, Pretoria, South Africa) (final concentration 2750 IU/ml), PPDb (Onderstepoort Biological Products) (final concentration 3300 IU/ml) and pokeweed mitogen (PWM; Sigma-Aldrich, St. Louis, MO, USA) (final concentration 10 µg/ml), were added to separate 2 mL microtubes (Eppendorf, Hamburg, Germany), respectively. For each set, 250 µl of heparinized whole blood was added to each tube. An additional 1 mL of whole blood was stimulated using the QuantiFERON®-TB Gold Plus TB2 tube (QFT; Qiagen, Venlo, The Netherlands), containing ESAT6/CFP10 antigens. Tubes were inverted several times and incubated at 37°C for 6 hours. Following incubation, whole blood in the QFT tube was transferred to a 2 mL microcentrifuge tube after which all tubes were centrifuged at 2 000 x g for 15 minutes. The plasma fraction was harvested, and the remaining cell pellet resuspended in 1 mL RNALater® Solution (Ambion, Foster City, CA, USA). The separated plasma, and cell pellets stabilized in RNALater®, were stored at -80°C until analyzed.

3.2.4. RNA extraction and reverse transcription

The RiboPure™-Blood Kit (Ambion) was used to extract RNA from RNALater® stabilized cell pellets, as previously described (de Waal *et al.*, 2021; Chapter 2). The first elution was performed using 30 µl elution buffer, incubated for 5 minutes at room temperature, and centrifuged for 30 seconds at 16 000 x g. The second elution was performed using 30 µl elution buffer, incubated for 3 minutes, and centrifuged for 1 minute at 16 000 x g to obtain a final volume of 60 µl total RNA. The quantity (ng) and A260/280 and A260/230 ratios of the extracted RNA were measured using the Nanodrop 1000 spectrophotometer (ThermoFisher Scientific, Wilmington, DE, USA), after which RNA was stored at -80°C prior to reverse transcription.

An estimated 200 ng of RNA was reverse transcribed to 20 µl of cDNA, using the QuantiTect® Reverse Transcription Kit (Qiagen, Hilden, Germany). To achieve this, genomic DNA (gDNA) Wipeout Buffer was added to ensure no gDNA contamination was carried over

from the extracted RNA, after which reverse transcription was performed according to manufacturer's instructions and cDNA stored at -20°C.

3.2.5. RT-qPCR

Reverse-transcriptase quantitative PCR (RT-qPCR) assays, previously developed and validated for African elephant whole blood RNA samples (de Waal *et al*., 2021; Chapter 2), were used to measure cytokine gene expression of *TNF*, *IL10*, and *TGFβ*, relative to the reference gene *GAPDH*, following antigen stimulation. Primers for each of the target and reference genes are listed in Table 3.1. Each gene target was amplified separately in a 10 µl PCR reaction containing 1 µl of cDNA, 5 µl iTAQ™ Universal SYBR® Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA), 0.4 µl of each forward and reverse primer (final concentration of 400 nM), and nuclease-free water. The qPCR cycling conditions were as follows: polymerase activation at 95°C for 30 seconds, followed by 40 cycles of denaturation at 95°C for 5 seconds, and annealing and elongation at 60°C for 30 seconds. Melt-curve analysis was performed over a 65–95°C range with increased increments of 0.5°C every 5 seconds. Standard melt curve analysis was used to confirm the specificity of RT-qPCRs. All samples were run on the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad).

Table 3.1: RT-qPCR primer sequences for reference (*GAPDH*) and target cytokine genes (*IL10, TGFβ,* and *TNF*) used to measure gene expression in African elephant whole blood following antigen stimulation.

Gene	Forward Primer (5' → 3')	Reverse Primer (5' → 3')	DM peak (°C)
GAPDH	ACGTGTCAGTGGTGGATCTG	GCCTGCTTCACCACCTTCTT	78.5
IL10	CGGTGGAGCAAGTGAAGGAA	TGTCAAACTCACTCATGGCC	77.5
TGFβ	TCCACCGAGAAGAACTGCTG	GCTCCAGATGTAAGGGCAGG	85
TNF	CCTGTAGCACACGTTGTAGC	CATCGGAAGGCACCATCAGC	82

DM - derivative melt curve peak

3.2.6. Data analysis

The relative fold change ($2^{-\Delta\Delta Cq}$) was used to measure abundance of the target transcript and up- or down-regulation of the target genes in response to antigen stimulation, which was calculated as previously described (Livak and Schmittgen, 2001). Up-regulation was classified as a minimum fold change ($2^{-\Delta\Delta Cq}$) of > 1, while fold change < 1 was classified as down-regulation (Radonic *et al.*, 2004; Schmittgen and Livak, 2008). To obtain the differential PPD response, fold change following PPDa stimulation was subtracted from fold change following PPDb expression ($2^{-\Delta\Delta Cq}$ PPDb - $2^{-\Delta\Delta Cq}$ PPDa). Thereafter, overall differences in gene expression in response to PPDa, PPDb, differential PPD response (PPDb-PPDa) and QFT-TB2 stimulation, were compared using the Friedman test. A Dunn's multiple comparison test was used to determine statistically significant differences following antigen stimulations, and a p-value < 0.05 was considered statistically significant. Statistical analyses were performed using GraphPad Prism version 8.0.0 for Windows, (GraphPad Software, San Diego, CA, USA; www.graphpad.com).

3.3. Results

Following RNA extraction, total RNA yield from the African elephant whole blood samples ranged from 174 to 2214 ng (median: 492 ng). The A260/280 ratio ranged from 1.7 to 2.2 (median: 2.0) and the A260/230 ratio ranged from 0.1 to 2.8 (median: 1.6). While A260/230 ratios fell outside the recommended range, all samples were included based on the limited availability of samples with all antigen stimulations.

Samples were opportunistically obtained from 11 African elephants from a *M. bovis*-endemic area for use in this study. Of the 11 African elephants, 10 were classified as MTBC uninfected based on serological and mycobacterial culture results, while 1 elephant was classified as MTBC seropositive, but mycobacterial culture negative. For each of the target genes (*TNF*, *IL10*, and *TGFβ*), the fold change observed for the seropositive (suspect immune sensitized) African elephant did not differ significantly from the ten uninfected African elephants, and this animal was therefore included in all data analyses going forward.

With the exception of responses to QFT-TB2 (ESAT6/CFP10) which were slightly down-regulated, elevated levels of *IL10* gene expression (median fold change) were observed following antigen (PPDa, PPDb) and PWM stimulation (Figure 3.1; Table 3.2). Slight down-regulation of *IL10* expression was also observed with the calculation of the differential PPD response. Significantly higher *IL10* gene expression was observed when PPDa and PPDb were compared to QFT-TB2 ($p = 0.0046$ and $p = 0.0002$, respectively); and PPDb was compared to the calculated differential PPD response ($p = 0.0121$). Two elephants had visibly higher *IL10* expression compared to the rest of the group following PPDa, PPDb, and PWM stimulation.

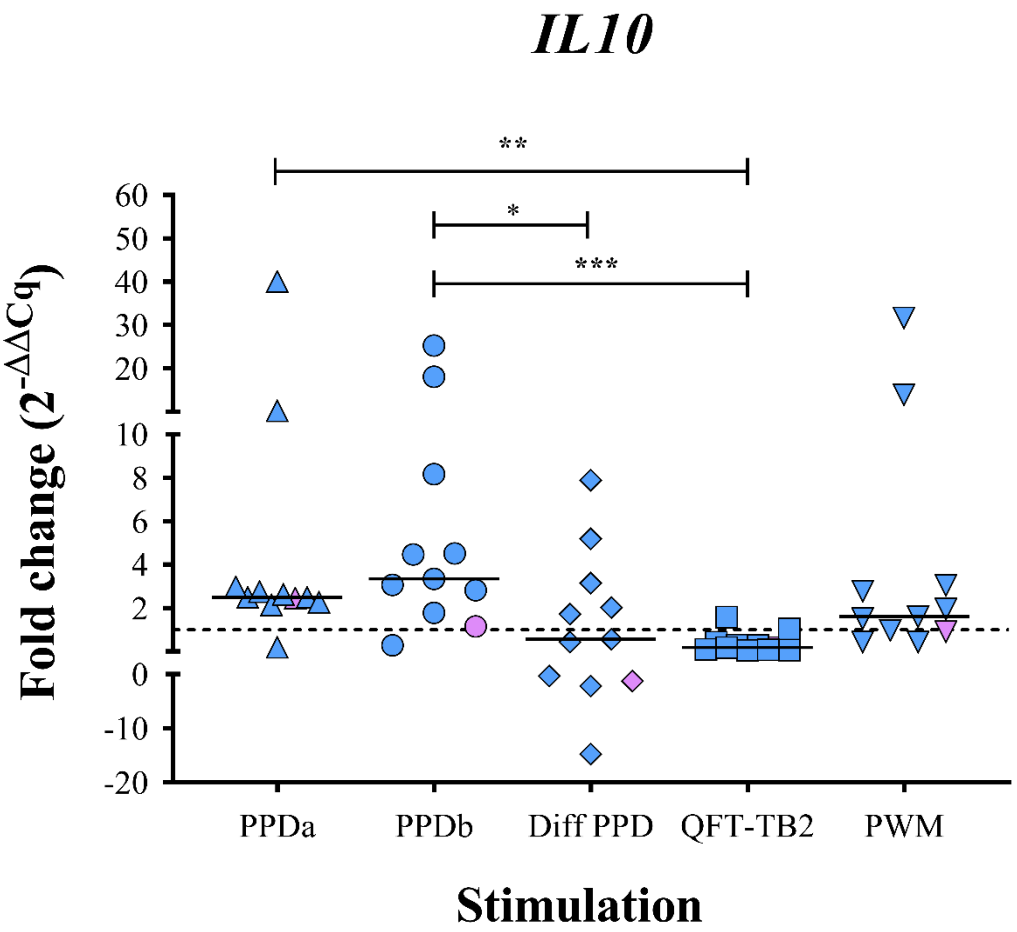


Figure 3.1: Gene expression of *IL10* measured in whole blood from 11 African elephants, following *M. avium* purified protein derivative (PPDa); *M. bovis* purified protein derivative (PPDb); early secretory antigenic target 6 kDa/culture filtrate protein 10 kDa (ESAT6/CFP-10; QFT-TB2) and pokeweed mitogen (PWM) stimulation, and calculation of the differential PPD response (PPDb-PPDa). Gene expression was measured following 6 hours of incubation. For each group, seronegative and mycobacterial culture negative elephants (n = 10) are indicated in blue; while one seropositive, mycobacterial culture negative elephant is indicated in purple. The median fold change of each group is indicated by a solid horizontal line. The dotted line on the y-axis indicates the minimum fold change ($2^{-\Delta\Delta Cq}$ >1) for expression to be considered up-regulated. * $p \leq 0.05$; ** $p \leq 0.01$; and *** $p \leq 0.001$.

Table 3.2: Gene expression for *IL10, TGFβ,* and *TNF*, calculated as median fold change (95% CI), following 6-hour stimulations of African elephant whole blood with *M. avium* purified protein derivative (PPDa); *M. bovis* purified protein derivative (PPDb); calculation of the differential PPD response (PPDb-PPDa); early secretory antigenic target 6 kDa/culture filtrate protein 10 kDa (ESAT6/CFP10; QFT-TB2) and pokeweed mitogen (PWM).

Gene	**Median Fold Change ($2^{-\Delta\Delta Cq}$)**				
	PPDa	**PPDb**	**Differential PPD (PPDb – PPDa)**	**QFT-TB2**	**PWM**
IL10	2.49	3.34	0.56	0.18	1.60
	(-1.25 - 14.10)	(1.33 - 11.90)	(-3.65 – 4.07)	(0.06 – 0.71)	(-1.01 – 11.80)
TGFβ	0.56	0.65	-0.02	0.55	0.20
	(0.041 – 1.15)	(0.31 – 1.65)	(-0.24 – 0.64)	(0.38 – 0.86)	(0.11 – 0.45)
TNF	0.65	0.62	0.04	0.34	0.40
	(-0.60 – 4.09)	(-0.10 – 4.25)	(-0.72 – 1.37)	(0.11 – 1.09)	(0.19 – 1.89)

Slight down-regulation of *TGFβ* expression was observed following all stimulations (Figure 3.2; Table 3.2). Despite low levels of gene expression, a significantly higher gene expression (median fold change) was observed when PPDa was compared to the calculated differential PPD response ($p = 0.005$); as well as when PPDb was compared to the calculated differential PPD response ($p = 0.012$) (Figure 3.2). One of the elephants which had high *IL10* responses to antigens, also had visibly higher *TGFβ* expression compared to the rest of the group following PPDa and PPDb stimulations.

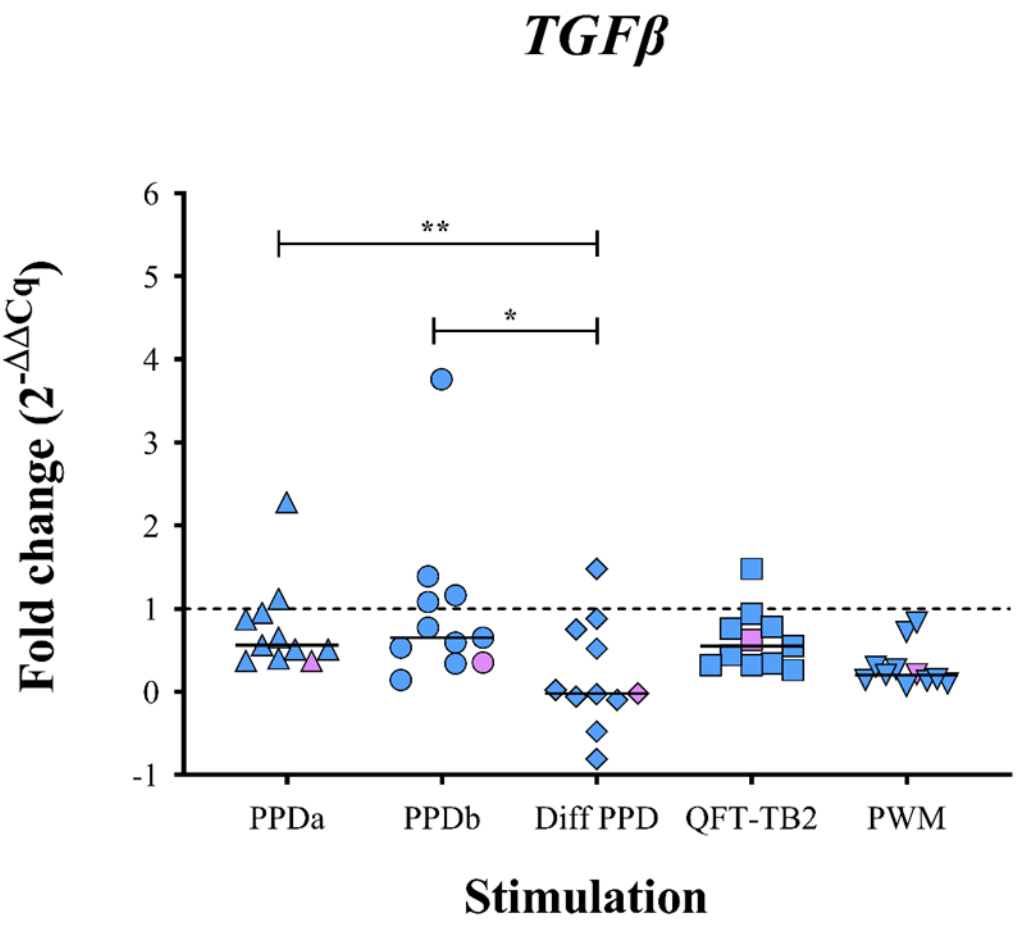


Figure 3.2: Gene expression for *TGFβ* measured in whole blood from 11 African elephants, following *M. avium* purified protein derivative (PPDa); *M. bovis* purified protein derivative (PPDb); early secretory antigenic target 6 kDa/culture filtrate protein 10 kDa (ESAT6/CFP-10; QFT-TB2) and pokeweed mitogen (PWM) stimulation, and calculation of the differential PPD response (PPDb-PPDa). Gene expression was measured following 6 hours of incubation. For each group, seronegative and mycobacterial culture negative elephants (n = 10) are indicated in blue; while one seropositive, mycobacterial culture negative elephant is indicated in purple. The median fold change of each group is indicated by a solid horizontal line. The dotted line on the y-axis indicates the minimum fold change ($2^{-\Delta\Delta Cq}$ >1) for expression to be considered up-regulated. * $p \leq 0.05$ and ** $p \leq 0.01$.

Like *TGFβ*, very slight down-regulation of *TNF* gene expression was observed following all stimulations (Figure 3.3; Table 3.2). The elephant with visibly higher *IL10* and *TGFβ* expression, also had visibly higher *TNF* expression compared to the rest of the group following PPDa, PPDb, and PWM stimulations. A second animal had visibly higher expression following PPDb and PWM stimulation, as well as the calculated PPD differential response. Again, despite low levels of gene expression, a significantly higher gene expression (median fold change) was

observed when PPDb was compared to the calculated differential PPD response (p = 0.005); as well as when PPDb was compared to QFT-TB2 (p = 0.03) (Figure 3.3).

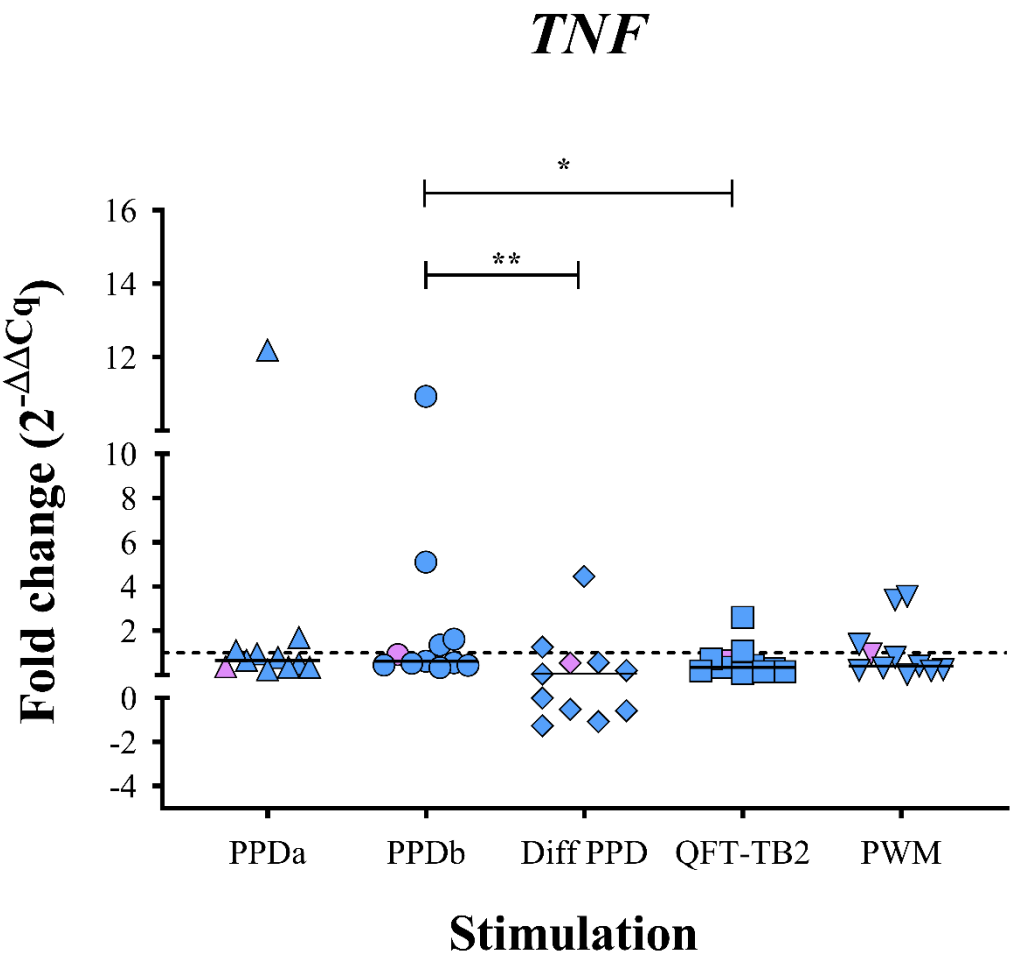


Figure 3.3: Gene expression of *TNF* measured in whole blood from 11 African elephants, following *M. avium* purified protein derivative (PPDa); *M. bovis* purified protein derivative (PPDb); early secretory antigenic target 6 kDa/culture filtrate protein 10 kDa (ESAT6/CFP-10; QFT-TB2) and pokeweed mitogen (PWM) stimulation, and calculation of the differential PPD response (PPDb-PPDa). Gene expression was measured following 6 hours of incubation. For each group, seronegative and mycobacterial culture negative elephants (n = 10) are indicated in blue; while one seropositive, mycobacterial culture negative elephant is indicated in purple. The median fold change of each group is indicated by a solid horizontal line. The dotted line on the y-axis indicates the minimum fold change ($2^{-\Delta\Delta Cq}$ >1) for expression to be considered up-regulated. * $p \leq 0.05$ and ** $p \leq 0.01$.

3.4. Discussion

Following the first fatal case of TB due to infection with *M. tb* in 2018 (Miller *et al.*, 2019) two additional elephants in KNP have been found to have incidental *M. bovis* infections (Goosen *et al.*, 2020a; Miller unpublished data). The occurrence of infection due to both *M. tb* and *M. bovis* raises concern as African elephants are ecologically and economically important for conservation (Blignaut, de Wit and Barnes, 2008; Kerley *et al.*, 2008). The TB cases have resulted in movement restrictions being placed on this population by DALRRD, to prevent possibly infected elephants from introducing MTBC into naïve populations through translocation (Miller *et al.*, 2019). In this chapter, levels of gene expression were measured following 6-hour TB antigen stimulation of heparinized whole blood collected from African elephants in KNP, to determine the use of a previously designed cytokine GEA (de Waal *et al.*, 2021; Chapter 2) for detection of MTBC sensitization.

Eleven African elephants from KNP were sampled for this study. Ten elephants were MTBC uninfected, based on serologic and mycobacterial culture results, while one elephant was classified as suspect immune sensitized due to a positive serological response, and a negative mycobacterial culture result. While mycobacterial culture is seen as the gold standard for TB diagnosis in elephants, it has also been shown to have varying sensitivity, with a positive serodiagnosis often being made years before mycobacterial culture confirmation (Lyashchenko *et al.*, 2006; Greenwald *et al.*, 2009). Thus, even though a negative mycobacterial culture result ruled out active shedding, the detection of antibodies to MTBC antigens was considered indicative of previous exposure.

Gene expression in the single suspect immune sensitized African elephant was comparable to gene expression (median fold change) observed for the MTBC uninfected animals. The lack of significantly different levels of gene expression in this elephant could be due to a difference in the dominant immune responses observed in various species, with elephants having a strong early humoral response, or may reflect an early infection that has not yet stimulated a cell-mediated immune response, or possibly a false positive serological result (Greenwald *et al.*, 2009; Miller *et al.*, 2018). It is also important to note that median fold change for all cytokines were below 2, except for *IL10* expression following PPDa and PPDb stimulation. This may indicate a lack of significant response to antigen stimulation for all samples, likely due to suboptimal incubation time, as 24 hours was previously identified as the optimal incubation time for African elephants (de Waal *et al.*, 2021; Chapter 2), or simply lack of immune

sensitization in these elephants. However, due to samples being collected prior to GEA optimization, only samples stimulated with antigens for 6 hours were available, which is one of the limitations of this study.

Low levels of up-regulation were observed for *IL10,* while median *TGFβ* and *TNF* expression showed very slight down-regulation, in whole blood samples following PWM stimulation. These immune responses, albeit low, may indicate the viability of the whole blood samples. Interestingly, these levels of expression were comparable with the lowest levels of expression in previous 6-hour cohorts (de Waal *et al*., 2021; Chapter 2). These overall expression levels were also visibly similar to the unstimulated, systemic immune responses observed in seronegative and seropositive elephants by Landolfi *et al.* (2010). These comparable expression levels between samples in this study and baseline immune responses in Asian elephants, may indicate suboptimal PWM stimulation, and therefore inadequate immune activation. This may be due to the suboptimal incubation time or poor response to PWM as the selected mitogen.

The anti-inflammatory cytokine *IL10* had the highest levels of gene expression, with up-regulation in elephant whole blood measured following PPDa and PPDb stimulation. These reactions are likely due to cross-reactivity to environmental non-tuberculous mycobacteria, since PPDs are a crude mixture of proteins with conserved homologous regions to various mycobacterial species (Borsuk *et al.*, 2009; Cho *et al.*, 2012). Expression following stimulation with the TB specific antigen ESAT6/CFP10 (QFT-TB2), as well as the calculated differential PPD response (specific TB response) showed slight down-regulation of *IL10*. The cytokine *IL10* has been shown to limit immune function during TB, and contribute to pathogenesis, therefore, down-regulation or lack of expression may be beneficial to help fight infection. However, a low or negligible response is also expected in an uninfected cohort (Redford, Murray and O'Garra, 2011).

Down-regulation of median *TGFβ* and *TNF* expression levels in whole blood following all antigen stimulations was observed, albeit at low levels. For *TGFβ,* the lowest level of expression was seen following calculation of the differential PPD response, although this may be an artifact of the calculation since the median fold changes for both PPDs were less than one. This may be expected in samples from uninfected African elephants. In Asian elephants, although not statistically significant, lower levels of *TGFβ* were measured in unstimulated peripheral whole blood samples of TB seropositive animals than in those that were seronegative

(Landolfi *et al.*, 2010). When Asian elephant PBMCs were stimulated with mycobacterial antigens, no significant difference in *TGFβ* expression was observed between TB-seropositive and seronegative animals (Landolfi *et al.*, 2014), which is similar to what was observed in our study. However, as samples from only one seropositive animal was available, definitive conclusions cannot be drawn.

A study done by Landolfi *et al.* (2010) showed higher expression levels of *TNF* in unstimulated peripheral whole blood samples of TB-seropositive elephants compared to seronegative elephants. Similarly, Landolfi *et al.* (2014) showed higher levels of *TNFα* mRNA extracted from PMBCs of seropositive Asian elephants versus seronegative elephants following 2-day stimulation with PPDb and CFP10, indicating its utility as a biomarker for TB. The overall down-regulation of *TNF* observed in our study might be due to the shorter 6-hour incubation, as the optimal time point for whole blood stimulation was previously determined to be 24 hours (de Waal *et al.*, 2021; Chapter 2). This suggests that a longer incubation period should be used with African elephant samples. Therefore, additional studies should be used to determine optimal time points for antigen stimulation.

A significant amount of individual variation among elephants was observed for cytokine gene expression following mitogen and antigen stimulations. Such variation presents another obstacle for interpretation of expression level differences between groups of seropositive and negative animals. Samples from three seronegative elephants had greater cytokine gene expression levels compared to the group. Elephant 6 had higher levels of expression for *IL10* and *TNF* following PPDa, PPDb and PWM stimulations, as well as higher levels of expression of *TGFβ* following PPDa and PPDb stimulations. As higher levels of expression were seen following PPDa stimulation as well as PWM, this heightened response is likely antigen non-specific. Similarly, in samples from elephant 8, higher levels of expression for *IL10* were observed following PPDa, PPDb, and PWM stimulations, also indicating non-specific expression. Elephant 7 had higher levels of *TNF* expression following PPDb and PWM stimulations, as well as the calculation of differential PPD response. However, there was no evidence that the responses in these elephants were due to TB specific immune sensitization.

Immune responses elicited by antigens PPDa and PPDb have been used to diagnose TB in species such as African buffalo and common warthogs (Roos *et al.*, 2016; Bernitz *et al.*, 2018a). A study by Landolfi *et al.* (2014) was able to differentiate between seronegative and TB seropositive animals based on *IFNγ, IL-12*, and *TNF* expression, using mRNA extracted from

PPDb and PPDa stimulated PBMC cultures. In the current study, PPDs were used to elicit a TB specific cytokine expression response for diagnosis in African elephants. However, as there was only one suspect immune sensitized animal, any differences between infected and uninfected African elephants could not be detected. In samples from the uninfected cohort, changes in cytokine gene expression following both PPDa and PPDb stimulations were observed, indicating responses to shared antigens, as PPDs contain a crude mixture of proteins (Cho *et al.*, 2012). When the target gene abundance was calculated as a differential PPD response (PPDb-PPDa), median gene expression levels of all three cytokines was comparable to those following QFT-TB2 stimulation. However, as mentioned previously, these antigen responses were also measured following suboptimal incubation times in elephants without evidence of specific TB immune sensitization, and future studies should investigate the measurement of gene expression following 24-hour stimulation of whole blood with these antigens in known immune sensitized animals.

3.5. Conclusion

The aim of this study was to measure levels of cytokine gene expression in African elephant blood following different mycobacterial antigen stimulation, to determine the potential utility of *IL10, TGFβ,* and *TNF* as biomarkers for MTBC infection. The up-regulation of *IL10,* and slight down-regulation of both *TNF* and *TGFβ* in mitogen-stimulated African elephant whole blood suggest that they should be investigated further for inclusion in a biomarker panel. However, additional experiments are required to confirm these findings. Samples were only available from ten elephants that did not have any evidence of MTBC exposure which limited the interpretation of results, and no definitive conclusions can be drawn regarding the use of these cytokine genes as potential biomarkers for TB. As the optimal time point for stimulation was previously determined as 24 hours (de Waal *et al*., 2021; Chapter 2), future studies will need to determine the levels of gene expression with longer incubation times for stimulation, and with confirmed MTBC infected African elephants to determine the use of these candidate biomarkers for the diagnosis of TB.

Chapter 4: General Discussion

This chapter provides a summary of our results and highlights the principle interpretations, with a focus on how these relate to the literature. In addition, limitations of the study are discussed.

African elephants are a charismatic species, however, there are large knowledge gaps regarding understanding of their immune system. The capability to evaluate immune responses is crucial for developing diagnostic tools as well as providing insight into comparative biology. Therefore, this project focused on investigating cell-mediated immune responses, specifically using cytokine gene expression, to identify biomarkers of immune activation and provide a foundation for techniques that can be applied to diagnostic test development.

The findings in this project have demonstrated that African elephant cytokine genes can be amplified using primers based on sequences from Asian elephants as well as other species, and resulted in the first report of full coding sequences for a number of cytokine (*CXCL9, CXCL10, IFNγ, IL4, IL12, TGFβ*) and reference (*ACTB, GAPDH, YWHAZ*) genes, as well as partial sequences (*B2M, IL10, TNF*) for this species. Using these sequences, African elephant specific primers were designed and used in RT-qPCR assays that could measure changes in gene abundance of cytokines *IL10*, *TNF*, *TGFβ*, normalized to a reference gene *GAPDH*, in whole blood stimulated with mitogen (PWM). The development of these GEAs then enabled a pilot study to determine if changes in cytokine gene expression in blood stimulated with mycobacterial antigens from elephants in a *M. bovis* endemic park, would provide evidence that these cytokines could be used as biomarkers for TB.

Mycobacterial infections, caused by *M. tb* and *M. bovis*, have been diagnosed in both captive and free-ranging Asian and African elephants (Mikota *et al.*, 2001; Lewerin *et al.*, 2005; Mikota, 2008; Angkawanish *et al.*, 2010; Verma-Kumar *et al.*, 2012; Obanda *et al.*, 2013; Perera *et al.*, 2014; Chandranaik *et al.*, 2017; Zachariah *et al.*, 2017; Miller *et al.*, 2019), showing that both are susceptible to this disease. However, very few reliable diagnostic tests are available for these species and may have suboptimal performance or other logistical challenges in obtaining results (Mikota *et al.*, 2001; Fowler, 2008; Paudel and Toshio, 2016; Hermes *et al.*, 2018). In addition, other than mycobacterial culture and PCR, the current tests (serology, IGRA) are based on detection of host immune responses and there has been limited research on the elephant immune system (Sreekumar *et al.*, 2007; Lyashchenko *et al.*, 2012;

Paudel *et al.*, 2016; Songthammanuphap *et al.*, 2020). Although limited studies on immune responses in Asian elephants have been reported (Landolfi *et al.*, 2009, 2010, 2014; Angkawanish *et al.*, 2013; Paudel and Toshio, 2016), there are fewer investigations of immune responses in African elephants (Greenwald *et al.*, 2009; Lyashchenko *et al.*, 2012; Edwards *et al.*, 2020). Thus, knowledge about the role of the immune system in health and disease, as well as diagnostic tools to assess these, are needed to improve our understanding, welfare, and facilitate conservation programs for these species. Consequently, this thesis described the development of novel cytokine GEAs to measure CMI responses in African elephants.

Cytokine GEAs and cytokine release assays measure immune mediators of CMI responses, which can be used to detect antigen-specific immune sensitization or general immune activation (using mitogens) *in vitro* after incubation of blood. These indirect immunological assays provide insights into the host's responses to different pathogens and are the basis of many blood-based diagnostic tests (Maas, Michel and Rutten, 2013; Clarke *et al.*, 2017; Chileshe *et al.*, 2019). Since cytokine protein detection often requires species-specific antibodies, limiting their use in novel species, cytokine GEAs are an alternative technique to measure CMI responses. This approach was selected to investigate *in vitro* CMI responses in African elephants in this project.

Cytokine GEAs have been used to detect mycobacterial infection in Asian elephants and various species (Higgitt *et al.*, 2017; Olivier *et al.*, 2017; Roos *et al.*, 2019), and have been shown to have high sensitivity in certain species (Maas, Michel and Rutten, 2013; Miller *et al.*, 2019). During the development of cytokine RT-qPCRs for African elephants, primer sequences were based on predicted African elephant mRNA transcripts as well as other species including Asian elephant and warthog, which allowed amplification and sequencing of the African elephant cytokine transcripts. This approach has been used when developing GEAs in novel species and is based on the conservation of cytokine sequences, especially in related organisms. For example, mRNA sequences of the domestic cat and dog were used to design primers for lion cytokine amplification (Olivier *et al.*, 2017), and domestic cat and dog, domestic ferret (*Mustela putorius furo*) and the giant panda (*Ailuropoda melanoleuca*) were used to amplify cytokine genes in the spotted hyena (Higgitt *et al.*, 2017). Therefore, the ability to design primers for PCR amplification of genes from a new species (African elephant) provides an approach for investigating comparative immunobiology of elephants.

Despite successful sequencing of nine cytokine and reference genes from African elephant blood cells, varying amplification led to only partial coding sequences for three genes *B2M*, *IL10*, and *TNF*. Possible SNPs located in the annealing regions may be the cause of varying amplification, as well as the possible formation of secondary structures which alter annealing (Derveaux, Vandesompele and Hellemans, 2010). These factors would also explain the inconsistent amplification of *CXCL9*, *CXCL10*, and *IFNγ*, using qPCR primers, as well as the out-of-range efficiency of *IL12*. It is also possible that species differences could influence the efficient amplification of *CXCL9*, *CXCL10*, and *IFNγ* since Asian elephant primers were used to initially amplify *IFNγ* for sequencing and warthog sequences used for *CXCL9* and *CXCL10*. While some temperature optimization was performed during this study, additional studies to optimize other PCR conditions and re-design of primers for these cytokines should be performed to improve results for GEAs that were unsuccessful.

In order to determine if the cytokine GEAs could detect immune activation in mitogen stimulated African elephant blood, real-time RT-qPCRs were developed and optimized. The reference gene *GAPDH* was identified as the most stable and efficiently expressed gene, and was compatible with *IL10, TGFβ,* and *TNF* for use as in the African elephant cytokine GEAs. The reference and target genes identified in African elephant blood were similar to those identified during the development of real-time RT-qPCRs for Asian elephants (Landolfi *et al.*, 2009). Because only three target genes met the initial criteria for qPCR development, these were used to investigate immune activation in African elephants.

The findings in a pilot study, to determine the optimal incubation time for cytokine gene expression following mitogen stimulation, revealed that expression levels for *IL10, TGFβ,* and *TNF* showed an unexpected high response measured in all three genes at 0 and 6 hours, followed by increased expression at 24 hours. Since blood samples were collected from free-ranging elephants for this study, the “fight-or-flight” response may have resulted in the early peak expression observed at the early time points. The release of catecholamines has been shown to induce both pro-inflammatory and anti-inflammatory gene expression in macrophages following physiological stress (Barnes, Carson and Nair, 2015; Shaw *et al.*, 2018). Alternatively, immune activation may have occurred during the handling of the samples and initial processing of the whole blood. However, since only three animals were used for the temporal expression component of this study, interpretations of results are limited. In the Asian elephant studies, blood was collected from trained animals, and RNA extracted from unstimulated samples, therefore, a comparison of responses could not be done (Landolfi *et al.*,

2009). This early response must be considered in future studies, and temporal cytokine gene expression responses should be measured in African elephants trained for blood collection to determine the significance of these findings.

Following identification of gene expression peaks after 6 and 24 hours of incubation, a cohort of 16 African elephants was used to investigate immune activation. Up-regulation of *TNF* expression was observed at both 6 and 24 hours, with gene expression at 24 hours being significantly higher. This high level of expression could indicate the importance of *TNF* to enhance immune responses in African elephants, as *TNF* is considered a master regulator of pro-inflammatory cytokine production, leading to the induction of *IL10, TGFβ,* and *IFNγ* (Wang, Czura and Tracey, 2003; Parameswaran and Patial, 2010). This cytokine has also been shown to be involved in granuloma formation during *M. tb* infection, and thus has an important role in activation of immune cells at the site of infection (Walzl *et al.*, 2011; Etna *et al.*, 2014). It also functions to inhibit differentiation of macrophages, induce apoptosis in mature T-cells, and enhance natural killer cell function (Rath and Aggarwal, 1999; Wang, Czura and Tracey, 2003), thus helping clear infection. The increased level of *TNF* expression in our study supports the potential use of this cytokine as a biomarker for immune activation in African elephants.

The two anti-inflammatory cytokines *IL10* and *TGFβ* showed variable expression in mitogen stimulated African elephant blood. Up-regulation of *IL10* expression was observed at both 6 and 24 hours after mitogen stimulation. This cytokine has been shown to inhibit the production of inflammatory cytokines and enhance activation, differentiation, and proliferation of B cells, which promotes humoral responses (Iyer and Cheng, 2012; Kriek, Areda and Didaba, 2019). As African elephants are known to display strong humoral responses to TB (Lyashchenko *et al.*, 2006, 2012; Greenwald *et al.*, 2009), *IL10* may play an important role in the shift towards a humoral response. During inflammation, IL10 inhibits the production of pro-inflammatory cytokines TNFα and IFNγ, to down-regulate the immune response and prevent tissue damage (Ouyang *et al.*, 2011; Redford, Murray and O'Garra, 2011; Ramani *et al.*, 2015). As *TNF* expression was also up-regulated following 24 hours of mitogen stimulation, the high level of *IL10* expression may be an antagonistic response to control inflammation.

Like *IL10,* the anti-inflammatory cytokine *TGFβ* plays a role in down-regulating immune responses to pathogens, and helps with tissue repair (Ramani *et al.*, 2015). Although *TGFβ* is known for its role in suppressing T-cell proliferation (Travis and Sheppard, 2014), it has also

been shown to have both proapoptotic and antiapoptotic effects (Howe, 2003). In this study, slight down-regulation of *TGFβ* expression was observed at 6 hours, while the median expression at 24 hours indicated no immune activation or a short-lived immune response, with a fold change of 1. This down-regulation of *TGFβ* could be mediated by the immune system to prevent suppression of T-cell responses and macrophage deactivation (Toossi and Ellner, 1998). As *TGFβ* is also known to induce *IL10* expression, the low level of *TGFβ* and the high level of *IL10* could indicate a shift in the inflammatory response.

The observations of up-regulation for *IL10* and down-regulation of *TNF* and *TGFβ* expression in mitogen stimulated African elephant blood were comparable with immune responses observed in unstimulated whole blood from Asian elephants (Landolfi *et al.*, 2010). Possible explanations for this could be suboptimal immune activation at these time points, or suboptimal induction of immune responses by PWM. In our study, PWM was the selected mitogen as it was previously shown to induce IFNγ in whole blood cultures of African elephants (Angkawanish *et al.*, 2013; Songthammanuphap *et al.*, 2020). Future studies should investigate the use of other mitogens such as Concanavalin A (ConA), phorbol myristate acetate plus ionomycin (PMA/I) or phytohaemagglutinin (PHA) which has been shown to induce IFNγ production in elephant PBMC cultures (Angkawanish *et al.*, 2013; Landolfi *et al.*, 2014; Songthammanuphap *et al.*, 2020). Another explanation could be that these cytokines do not play an important role in immune responses in elephants. Thus, further investigation into the role of these genes in the African elephant immune system is required and no definitive conclusions can be drawn regarding gene expression in this species. Finally, although gene expression was used as an indication of cell viability, future studies should consider using trypan blue exclusion assays to determine cell viability (Landolfi *et al.*, 2014).

Since the goal of this project was to develop cytokine GEAs as a step toward a diagnostic assay for TB in African elephants, samples were collected from animals in a *M. bovis* endemic park to explore the proof of concept that these assays could differentiate between MTBC immune sensitized and uninfected individuals. However, samples from only one suspect immune sensitized African elephant (based on negative mycobacterial culture and positive serological responses) were available. Since this elephant had antigen-specific cytokine responses comparable with those of the uninfected elephants, it was subsequently analyzed as part of the uninfected cohort. The low level of *IL10, TGFβ* and *TNF* expression in the suspect sensitized elephant may be indicative of an early infection, with CMI responses not yet being stimulated, or lack of response due to false positive serologic test. Elephants also have a strong

early humoral response, which may also influence the levels of gene expression observed in this elephant (Greenwald *et al.*, 2009; Miller *et al.*, 2018). Even though none of the elephants were confirmed to be MTBC immune sensitized, there was a high level of *IL10* expression in response to stimulation with antigens PPDa and PPDb in several individual elephants, which demonstrates that cytokine responses can be detected in antigen-stimulated elephant blood samples. However, this up-regulation was likely due to cross-reactivity with environmental mycobacteria that share antigens with MTBC (Borsuk *et al.*, 2009; Cho *et al.*, 2012). Overall, the negligible change in cytokine expression in cells stimulated with specific mycobacterial peptides (QFT-TB2) was expected in uninfected elephants.

In Asian elephants, no statistically significant difference in *TGFβ* expression was observed between TB seropositive and seronegative cohorts following antigen stimulation of PBMC cultures (Landolfi *et al.*, 2014). However, higher levels of *TNFα* expression were measured in unstimulated peripheral whole blood samples (Landolfi *et al.*, 2010); as well as mRNA extracted from antigen stimulated PMBCs of seropositive versus seronegative Asian elephants. Although not significant, baseline expression of *TNFα*, *IFNγ*, and *IL4* tended to be higher, and *TGFβ, IL10*, and *IL12* lower in the TB seropositive Asian elephants (Landolfi *et al.*, 2010). Further studies should investigate responses in known immune sensitized/infected elephants to identify cytokines that can be used as biomarkers for TB.

There were several limitations encountered in this study. Since elephant samples were collected opportunistically from immobilized animals, the number of samples and conditions for processing were determined prior to the initiation of the project. A limited number of African elephants were used for sequencing of mRNA transcripts (n = 2), as well as the measurement of temporal immune responses to PWM stimulation (n = 3). During the development of the real-time RT-qPCRs, the unsuccessful amplification of *IFNγ, CXCL9, CXCL10*, inefficient amplification of *IL12*, and the non-compatibility of *IL4* with *GAPDH* may be a result of flaws in primer design or lack of optimization of PCR conditions. In addition, the selection of PWM as the mitogen may not be ideal for immune activation of African elephant whole blood. The absence of a MTBC culture confirmed infected African elephant prevented assessment of antigen-specific cytokine gene expression for identification of candidate biomarkers for TB detection. Thus, future studies will need to investigate the use of *IL10*, *TNF*, and *TGFβ,* using confirmed infected and uninfected African elephants, to determine optimal incubation time points, mitogen selection, additional cytokine genes, to assess whether these cytokines can be used in a biomarker panel for TB detection in this species.

Chapter 5: General Conclusion

In this chapter, the overall contributions of this study's findings to the understanding of immune responses in African elephants are highlighted. Recommendations for future research are also included.

The characterization of immune responses is important to understanding factors that affect the health and welfare of African elephants, and thus their conservation. Knowledge of and tools to assess immune responses can facilitate diagnosis and strategies for disease management. Since there is a paucity of information on the African elephant immune system, this study aimed to identify biomarkers of immune activation, as well as immune sensitization to MTBC infection, as a first step towards development of diagnostic tools to improve disease detection and surveillance.

To our knowledge, this is the first study to investigate cytokine responses in African elephants using mRNA extracted from stimulated whole blood. During this study, Asian elephant and common warthog primers were modified to successfully amplify the first confirmed cytokine and reference gene mRNA transcripts for African elephants. The resulting transcripts contained full and partial coding sequences and showed high identity to Asian elephant (96.6-99.8%) and common warthog (72-97.6%) mRNA transcripts for these cytokines. Future studies should consider the use of reagents designed for Asian elephant and other species to detect immune responses in African elephants. The generated African elephant transcripts facilitated the design of species-specific primers for RT-qPCR amplification, and the development of real-time RT-qPCRs for *IL10, TGFβ,* and *TNF*. These RT-qPCRs will provide an important foundation for development of future assays in this species as well as studies on comparative immunobiology.

Findings from this study demonstrated that cytokine gene expression assays show promise for detecting immune activation in African elephant whole blood. However, only three target genes (*IL10, TGFβ,* and *TNF*) and the reference gene *GAPDH*, were determined to meet the criteria for the development of the GEA. Although other African elephant cytokine and reference genes were sequenced, additional optimization will be required to develop a more robust panel of GEAs and identify biomarkers for detection of antigen-specific immune responses in African elephants.

Cytokine expression in mycobacterial antigen stimulated African elephant samples revealed up-regulation of *IL10* in response to complex mixtures of purified protein derivative from *M. avium* and *M. bovis*, suggesting that these elephants were sensitized to environmental antigens. However, since none of the elephants were known to be infected with MTBC, it was not possible to determine if these GEAs could distinguish between African elephants that were immune sensitized or not.

The GEAs developed in this study included both pro-inflammatory and anti-inflammatory cytokines which have been associated with detection of and immunity to MTBC infection. The balance of T_H1 (cell-mediated) and T_H2 (humoral) responses is important in determining outcome, although cytokine profiles can vary by species (Rhodes *et al.*, 2000, 2011; Thacker, Palmer and Waters, 2006; Cavalcanti *et al.*, 2012). Since elephants show a robust humoral response to TB (Greenwald *et al.*, 2009), it would not be unexpected that they also have a unique cytokine profile. Therefore, further investigation of cytokine gene expression in African elephants may provide insight into their immunologic response patterns, which could inform novel diagnostic tests.

Findings in this project as well as other recent studies have shown that use of reagents and assays developed for other species, such as cytokine primers and ELISA antibodies, can be adapted for use with elephant blood (Landolfi *et al.*, 2009; Edwards *et al.*, 2020). Therefore, this may provide a more efficient approach to studying immune responses in these species. For example, the Equine RT^2 Profiler PCR array (Qiagen) has been used to screen RNA extracted from QuantiFERON® TB Gold Plus (In-Tube) (QFT; Qiagen) stimulated whole blood from *M. bovis*-infected white rhinoceros to identify candidate biomarkers for TB (Chileshe *et al.*, 2021). A similar study using either RNA or plasma from QFT stimulated elephant whole blood could be performed for biomarker discovery. This would significantly advance insight into the elephant immune system and comparative immunobiology of responses to TB.

Future studies should be designed to address the limitations of this study and use findings to advance knowledge of the role of cytokines in African elephant immune responses. Larger sample sizes are needed to confirm cytokine sequences and investigate possible SNPs between African elephant transcripts. The investigation of temporal responses should also include a larger study population, ideally including samples from trained elephants to determine the influence of stress on immune responses. Identification of known MTBC infected elephants would facilitate studies to identify candidate biomarkers for diagnostic test development.

Collaborations established during this study, between local and international researchers, as well as governmental and private organizations (SANParks, SANBI-NZG, State Veterinarians from DALRRD, and veterinarians at private wildlife reserves), can potentially provide access to diverse African elephant populations. Finally, other aspects, such as antibody responses should be investigated for complete understanding of immune responses in this species and increase knowledge of TB responses. The cytokine GEAs developed in this study are the first step in identifying biomarkers that could be assessed using cytokine release in plasma from stimulated whole blood, which will add another tool to studying African elephant immunology.

References

Abbas, A. K., Lichtman, A. H. and Pillai, S. (eds) (2016) 'Innate Immunity: The Early Defense Against Infections', in *Basic Immunology: Functions and Disorders of the Immune System*. 5th edn. Missouri, USA: Elsevier Inc., pp. 27–53. doi: 978-1-4557-0707-2.

Abegglen, L. M., Fuery, A., Kiso, W. K., Schmitt, D. L., Ling, P. D. and Schiffman, J. D. (2018) 'Mammalia: Proboscidea: Elephant Immune System', in Cooper, E. L. (ed.) *Advances in Comparative Immunology*. 1st edn. Springer International Publishing AG, pp. 863–883. doi: 10.1007/978-3-319-76768-0_24.

Altschuk, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman, D. J. (1990) 'Basic Local Alignment Search Tool', *Journal of Molecular Biology*, 215, pp. 403–410. doi: 10.1016/S0022-2836(05)80360-2.

Andersen, C. L., Jensen, J. L. and Ørntoft, T. F. (2004) 'Normalization of Real-Time Quantitative Reverse Transcription-PCR Data: A Model-Based Variance Estimation Approach to Identify Genes Suited for Normalization, Applied to Bladder and Colon Cancer Data Sets', *Cancer Research*, 64(15), pp. 5245–5250. doi: 10.1158/0008-5472.CAN-04-0496.

Anderson, P., Phillips, K., Stoecklin, G. and Kedersha, N. (2004) 'Post-Transcriptional Regulation of Proinflammatory Proteins', *Journal of Leukocyte Biology*, 76, pp. 42–47. doi: 10.1189/jlb.1103536.

Angkawanish, T., Morar, D., van Kooten, P., Bontekoning, I., Schreuder, J., Maas, M., Wajjwalku, W., Sirimalaisuwan, A., Michel, A., Tijhaar, E. and Rutten, V. (2013) 'The Elephant Interferon Gamma Assay: A Contribution to Diagnosis of Tuberculosis in Elephants', *Transboundary and Emerging Diseases*, 60, pp. 53–59. doi: 10.1111/tbed.12098.

Angkawanish, T., Wajjwalku, W., Sirimalaisuwan, A., Mahasawangkul, S., Kaewsakhorn, T., Boonsri, K. and Rutten, V. P. M. G. (2010) '*Mycobacterium tuberculosis* Infection of Domesticated Asian Elephants, Thailand', *Emerging Infectious Diseases*, 16(12), pp. 1949–1951. doi: 10.3201/eid1612.100862.

Barnes, M. A., Carson, M. J. and Nair, M. G. (2015) 'Non-Traditional Cytokines: How Catecholamines and Adipokines Influence Macrophages in Immunity, Metabolism and the Central Nervous System', *Cytokine*, 72(2), pp. 210–219. doi: 10.1016/j.cyto.2015.01.008.

Bass, K. E., Nonnecke, B. J., Palmer, M. V, Thacker, T. C., Hardegger, R., Schroeder, B., Raeber, A. J. and Water, W. R. (2013) 'Clinical and Diagnostic Developments of a Gamma Interferon Release Assay for Use in Bovine Tuberculosis Control Programs', *Clinical and Vaccine Immunology*, 20(12), pp. 1827–1835. doi: 10.1128/CVI.00519-13.

Beamer, G. L., Flaherty, D. K., Assogba, B. D., Stromberg, P., Gonzalez-Juarrero, M., de Waal Malefyt, R., Vesosky, B. and Turner, J. (2008) 'Interleukin-10 Promotes *Mycobacterium tuberculosis* Disease Progression in CBA/J Mice', *The Journal of Immunology*, 181(8), pp. 5545–5550. doi: 10.4049/jimmunol.181.8.5545.

Benson, D. A., Cavanaugh, M., Clark, K., Karsch-Mizrachi, I., Lipman, D. J., Ostell, J. and Sayers, E. W. (2013) 'GenBank', *Nucleic Acids Research*, 41, pp. 36–42. doi: 10.1093/nar/gks1195.

Bernitz, N., Clarke, C., Roos, E. O., Goosen, W. J., Cooper, D., van Helden, P. D., Parsons, S. D. C. and Miller, M. A. (2018a) 'Detection of *Mycobacterium bovis* Infection in African Buffaloes (*Syncerus caffer*) using QuantiFERON®-TB Gold (QFT) Tubes and the Qiagen Cattletype® IFN-gamma ELISA', *Veterinary Immunology and Immunopathology*. Elsevier, 196, pp. 48–52. doi: 10.1016/j.vetimm.2017.12.010.

Bernitz, N., Goosen, W. J., Clarke, C., Kerr, T. J., Higgitt, R., Roos, E. O., Cooper, D. V., Warren, R. M., van Helden, P. D., Parsons, S. D. C. and Miller, M. A. (2018b) 'Parallel Testing Increases Detection of *Mycobacterium bovis*-Infected African Buffaloes (*Syncerus caffer*)', *Veterinary Immunology and Immunopathology*. Elsevier, 204, pp. 40–43. doi: 10.1016/j.vetimm.2018.09.004.

Bernitz, N., Kerr, T. J., de Waal, C., Cooper, D. V., Warren, R. M., Van Helden, P. D., Parsons, S. D. C. C. and Miller, M. A. (2020) 'Test Characteristics of Assays to Detect *Mycobacterium bovis* Infection in High-Prevalence African Buffalo (*Syncerus caffer*) Herds', *Journal of Wildlife Diseases*, 56(2), pp. 462–465. doi: 10.7589/2019-06-173.

Blanc, J. (2008) *Loxodonta africana, The IUCN Red List of Threatened Species*. IUCN Global Species Programme Red List Unit. Available at: https://nc.iucnredlist.org/redlist/species-of-the-day/loxodonta-africana/pdfs/original/loxodonta-africana.pdf (Accessed: 22 January 2018).

Blignaut, J., de Wit, M. and Barnes, J. (2008) 'The Economic Value of Elephants', in Scholes,

R. J. and Mennell, K. G. (eds) *Elephant Management: A Scientific Assessment for South Africa*. 1st edn. Johannesburg, SA: Wits University Press, pp. 446–476. doi: 10.18772/22008034792.21.

Borsuk, S., Newcombe, J., Mendum, T. A., Dellagostin, O. A. and McFadden, J. (2009) 'Identification of Proteins from Tuberculin Purified Protein Derivative (PPD) by LC-MS/MS', *Tuberculosis*, 89, pp. 423–430. doi: 10.1016/j.tube.2009.07.003.

Branco, P. S., Merkle, J. A., Pringle, R. M., Pansu, J., Potter, A. B., Reynolds, A., Stalmans, M. and Long, R. A. (2019) 'Determinants of Elephant Foraging Behaviour in a Coupled Human-Natural System: Is Brown the New Green?', *Journal of Animal Ecology*, 88, pp. 780–792. doi: https://doi.org/10.5061/dryad.6v5v88g.

Bronson, E., McClure, M., Sohl, J., Wiedner, E., Cox, S., Latimer, E. M., Pearson, V. R., Hayward, G. S., Fuery, A. and Ling, P. D. (2017) 'Epidemiologic Evaluation of Elephant Endotheliotropic Herpesvirus 3B Infection in an African Elephant (*Loxodonta africana*)', *Journal of Zoo and Wildlife Medicine*, 48(2), pp. 335–343. doi: https://doi.org/10.1638/2016-0063R.1.

Cavalcanti, Y. V. N., Brelaz, M. C. A., Neves, J. K. de A., Ferraz, J. C. and Pereira, V. R. A. (2012) 'Role of TNF-Alpha, IFN-Gamma, and IL-10 in the Development of Pulmonary Tuberculosis', *Pulmonary Medicine*, 2012(745483), pp. 1–10. doi: 10.1155/2012/745483.

Chandranaik, B. M., Shivashankar, B. P., Umashankar, K. S., Nandini, P., Giridhar, P., Byregowda, S. M., Shrinivasa, B. M., Zachariah, A., Pandiyan, J., Madhavilatha, G. K., Mundayoor, S., Chandramohan, B., Sajesh, P. K., Santhosh, S. and Mikota, S. K. (2017) '*Mycobacterium tuberculosis* Infection in Free-Roaming Wild Asian Elephant', *Emerging Infectious Diseases*, 23(3), pp. 555–557. doi: 10.3201/eid2303.161439.

Chileshe, J., Roos, E. O., Goosen, W. J., Buss, P., Hausler, G., Rossouw, L., Manemela, T., van Helden, P., Warren, R., Parsons, S. D. C. and Miller, M. (2019) 'An Interferon-Gamma Release Assay for the Diagnosis of the *Mycobacterium bovis* Infection in White Rhinoceros (*Ceratotherium simum*)', *Veterinary Immunology and Immunopathology*, 217(109931), pp. 1–4. doi: 10.1016/j.vetimm.2019.109931.

Chileshe, J., Kerr, T. J., Kinnear, C., Buss, P. E., van Helden, P. D., Warren, R. M., Miller, M.

A. and Parson, S. D. C. (2021) 'Cytokine Biomarker Discovery in the White Rhinoceros (*Ceratotherium simum*)', *Veterinary Immunology and Immunopathology*, 232(110168), pp. 1–6. doi: 10.1016/j.vetimm.2020.110168.

Cho, Y. S., Dobos, K. M., Prenni, J., Yang, H., Hess, A., Rosenkrands, I., Andersen, P., Ryoo, S. W., Bai, G.-H., Brennan, M. J., Izzo, A., Bielefeldt-Ohmann, H. and Belisle, J. T. (2012) 'Deciphering the Proteome of the *In Vivo* Diagnostic Reagent "Purified Protein Derivative" from *Mycobacterium tuberculosis*', *Proteomics*, 12, pp. 979–991. doi: 10.1002/pmic.201100544.

Clarke, C., Patterson, S. J., Drewe, J. A., David Van Helden, P., Miller, M. A., David, S., Parsons, C., Helden, P. D., Miller, M. A. and Parsons, S. D. C. (2017) 'Development and Evaluation of a Diagnostic Cytokine-Release Assay for *Mycobacterium suricattae* Infection in Meerkats (*Suricata suricatta*)', *BMC Veterinary Research*, 13(1), pp. 1–6. doi: 10.1186/s12917-016-0927-x.

Coscolla, M. and Gagneux, S. (2014) 'Consequences of Genomic Diversity in *Mycobacterium tuberculosis*', *Seminars in Immunology*, 26(6), pp. 431–444. doi: 10.1016/j.smim.2014.09.012.

Cousins, D. V and Florisson, N. (2005) 'A Review of Tests Available for Use in the Diagnosis of Tuberculosis in Non-bovine Species', *Scientific and Technical Review of the Office International des Epizooties (Paris)*, 24(3), pp. 1039–1059. doi: 10.20506/rst.24.3.1635

Cunningham, F., Achuthan, P., Akanni, W., Allen, J., Amode, M. R., Armean, I. M., Bennett, R., Bhai, J., Billis, K., Boddu, S., Cummins, C., Davidson, C., Dodiya, K. J., Gall, A., Girón, C. G., Gil, L., Grego, T., Haggerty, L., Haskell, E., Hourlier, T., Izuogu, O. G., Janacek, S. H., Juettemann, T., Kay, M., Laird, M. R., Lavidas, I., Liu, Z., Loveland, J. E., Marugán, J. C., Maurel, T., McMahon, A. C., Moore, B., Morales, J., Mudge, J. M., Nuhn, M., Ogeh, D., Parker, A., Parton, A., Patricio, M., Abdul Salam, A. I., Schmitt, B. M., Schuilenburg, H., Sheppard, D., Sparrow, H., Stapleton, E., Szuba, M., Taylor, K., Threadgold, G., Thormann, A., Vullo, A., Walts, B., Winterbottom, A., Zadissa, A., Chakiachvili, M., Frankish, A., Hunt, S. E., Kostadima, M., Langridge, N., Martin, F. J., Muffato, M., Perry, E., Ruffier, M., Staines, D. M., Trevanion, S. J., Aken, B. L., Yates, A. D., Zerbino, D. R., Flicek, P., Gall, A., Garc, C., Haskell, E., Hourlier, T., Izuogu, O. G., Janacek, S. H., Juettemann, T., Kay, M., Laird, M. R., Lavidas, I., Liu, Z., Marug, C., Loveland, J. E., Maurel, T., McMahon, A. C., Moore, B., Morales, J., Mudge, J. M., Nuhn, M., Ogeh, D., Parker, A., Parton, A., Patricio, M., Abdul, A.

I., Schmitt, B. M., Schuilenburg, H., Sheppard, D., Sparrow, H., Stapleton, E., Szuba, M., Taylor, K., Threadgold, G., Thormann, A., Vullo, A., Walts, B., Winterbottom, A., Zadissa, A., Chakiachvili, M., Frankish, A., Hunt, S. E., Kostadima, M., Langridge, N., Martin, F. J., Muffato, M., Perry, E., Ruffier, M., Staines, D. M., Trevanion, S. J., Aken, B. L., Yates, A. D., Zerbino, D. R. and Flicek, P. (2019) 'Ensembl 2019', *Nucleic Acids Research*, 47(D1), pp. D745–D751. doi: 10.1093/nar/gky1113.

Derveaux, S., Vandesompele, J. and Hellemans, J. (2010) 'How To Do Successful Gene Expression Analysis Using Real-Time PCR', *Methods*, 50(4), pp. 227–230. doi: 10.1016/j.ymeth.2009.11.001.

Dhanasekaran, S., Jenum, S., Stavrum, R., Ritz, C., Faurholt-Jepsen, D., Kenneth, J., Vaz, M., Grewal, H. M. S. and Doherty, T. M. (2013) 'Identification of Biomarkers for *Mycobacterium tuberculosis* Infection and Disease in BCG-Vaccinated Young Children in Southern India', *Genes and Immunity*, 14(6), pp. 356–364. doi: 10.1038/gene.2013.26.

de Waal, C. R., Kleynhans, L., Parsons, S. D., Goosen, W. J., Hausler, G., Buss, P. E., Warren, R. M., van Helden, P. D., Landolfi, J. A., Miller, M. A. and Kerr, T. J. (2021) 'Development of a Cytokine Gene Expression Assay for the Relative Quantification of the African Elephant (*Loxodonta africana*) Cell-Mediated Immune Responses', *Cytokine*, 141(155453), pp. 1–8. doi: 10.1016/j.cyto.2021.155453.

Edwards, K. L., Miller, M. A., Siegal-Willott, J. and Brown, J. L. (2020) 'Serum Health Biomarkers in African and Asian Elephants: Value Ranges and Clinical Values Indicative of the Immune Response', *Animals*, 10. doi: 10.3390/ani10101756.

Etna, M. P., Giacomini, E., Severa, M. and Coccia, E. M. (2014) 'Pro-and Anti-Inflammatory Cytokines in Tuberculosis: A Two-Edged Sword in TB Pathogenesis', *Seminars in Immunology*, 26, pp. 543–551. doi: 10.1016/j.smim.2014.09.011.

Fowler, M. E. (2008) 'Infectious Diseases', in Fowler, M. E. and Mikota, S. K. (eds) *Biology, Medicine and Surgery of Elephants*. 1st edn. Oxford, United Kingdom: Blackwell Publishing Ltd, pp. 138–158. doi: 10.1002/9780470344484.ch11.

Goosen, W. J., Miller, M. A., Chegou, N. N., Cooper, D., Warren, R. M., van Helden, P. D. and Parsons, S. D. C. C. (2014) 'Agreement Between Assays of Cell-Mediated Immunity

Utilizing *Mycobacterium bovis*-Specific Antigens for the Diagnosis of Tuberculosis in African Buffaloes (*Syncerus caffer*)', *Veterinary Immunology and Immunopathology*, 160, pp. 133–138. doi: 10.1016/j.vetimm.2014.03.015.

Goosen, W. J., Kerr, T. J., Kleynhans, L., Buss, P., Cooper, D., Warren, R. M., Helden, P. D. Van, Schröder, B., Parsons, S. D. C. and Miller, M. A. (2020a) 'The VetMAX™ *M. tuberculosis* Complex PCR Kit Detects MTBC DNA in Antemortem and Postmortem Samples from White Rhinoceros (*Ceratotherium simum*), African Elephants (*Loxodonta africana*) and African Buffaloes (*Syncerus caffer*)', *BMC Veterinary Research*, 16(220), pp. 16–21. doi: 10.1186/s12917-020-02438-9.

Goosen, W. J., Kerr, T. J., Kleynhans, L., Warren, R. M., Helden, P. D. Van, Persing, D. H., Parsons, S. D. C., Buss, P. and Miller, M. A. (2020b) 'The Xpert MtB/Rif Ultra Assay Detects *Mycobacterium tuberculosis* Complex DNA in White Rhinoceros (*Ceratotherium simum*) and African Elephants (*Loxodonta africana*)', *Scientific Reports*, 10(14482), pp. 1–7. doi: 10.1038/s41598-020-71568-9.

Greenwald, R., Lyashchenko, O., Esfandiari, J., Miller, M., Mikota, S., Olsen, J. H., Ball, R., Dumonceaux, G., Schmitt, D., Moller, T., Payeur, J. B., Harris, B., Sofranko, D., Waters, W. R. and Lyashchenko, K. P. (2009) 'Highly Accurate Antibody Assays for Early and Rapid Detection of Tuberculosis in African and Asian Elephants', *Clinical and Vaccine Immunology*, 16(5), pp. 605–612. doi: 10.1128/CVI.00038-09.

Han, Z., Kang, D., Joo, Y., Lee, J., Oh, G., Choi, S., Ko, S., Je, S., Choi, H. J. and Song, J. J. (2018) '*TGF-β* Downregulation-Induced Cancer Cell Death is Finely Regulated by the SAPK Signaling Cascade', *Experimental & Molecular Medicine*, 50(162), pp. 1–19. doi: 10.1038/s12276-018-0189-8.

Hermes, R., Saragusty, J., Moser, I., Holtze, S., Nieter, J., Sachse, K., Voracek, T., Bernhard, A., Bouts, T., Göritz, F. and Hildebrandt, T. B. (2018) 'Bronchoalveolar Lavage for Diagnosis of Tuberculosis Infection in Elephants', *Epidemiology and Infection*, 146(4), pp. 481–488. doi: 10.1017/S0950268818000122.

Higgins, D. M., Sanchez-Campillo, J., Rosas-Taraco, A. G., Lee, E. J., Orme, I. M. and Gonzalez-Juarrero, M. (2009) 'Lack of *IL-10* Alters Inflammatory and Immune Responses During Pulmonary *Mycobacterium tuberculosis* Infection', *Tuberculosis*, 89(2), pp. 149–157.

doi: 10.1016/j.tube.2009.01.001.

Higgitt, R. L., Buss, P. E., Van Helden, P. D., Miller, M. A. and Parsons, S. D. C. (2017) 'Development of Gene Expression Assays Measuring Immune Responses in the Spotted Hyena (*Crocuta crocuta*)', *African Zoology*, 52(2), pp. 99–104. doi: 10.1080/15627020.2017.1309300.

Howe (2003) 'Transforming Growth Factor β', in Thomson, A. W. and Lotze, M. T. (eds) *The Cytokine Handbook*. 4th edn. London, UK: Elsevier Science Ltd, pp. 1119–1152. doi: 10.1016/B978-012689663-3/50018-1.

Iyer, S. S. and Cheng, G. (2012) 'Role of Interleukin 10 Transcriptional Regulation in Inflammation and Autoimmune Disease', *Critical Review in Immunology*, 32(1), pp. 23–63. doi: 10.1615/critrevimmunol.v32.i1.30

Kamerbeek, J., Schouls, L., Kolk, A., Van Agterveld, M., Van Soolingen, D., Kuijper, S., Bunschoten, A., Molhuizen, H., Shaw, R., Goyal, M. and Van Embden, J. (1997) 'Simultaneous Detection and Strain Differentiation of *Mycobacterium tuberculosis* for Diagnosis and Epidemiology', *Journal of Clinical Microbiology*, 35(4), pp. 907–914. doi: 10.1128/jcm.35.4.907-914.1997.

Keet, D. F., Michel, A. L., Bengis, R. G., Becker, P., van Dyk, D. S., van Vuuren, M., Rutten, V. P. M. G. and Penzhorn, B. L. (2010) 'Intradermal Tuberculin Testing of Wild African Lions (*Panthera leo*) Naturally Exposed to Infection with *Mycobacterium bovis*', *Veterinary Microbiology*, 144(3–4), pp. 384–391. doi: 10.1016/j.vetmic.2010.01.028.

Kehrl, J. H., Roberts, A. B., Wakefield, L. M., Jakowlew, S., Sporn, M. B. and Fauci, A. S. (1986) 'Transforming Growth Factor Beta is an Important Immunomodulatory Protein for Human B Lymphocytes.', *The Journal of Immunology*, 137(12), pp. 3855–3860. doi: 0022-1767/86/13712-3855S02.00/0.

Kerley, G. I. H., Landman, M., Kruger, L., Owen-Smith, N., Balfour, D., de Boer, W. F., Gaylard, A., Lindsay, K. and Slotow, R. (2008) 'Effects of Elephants on Ecosystems and Biodiversity', in Scholes, R. J. and Mennell, K. G. (eds) *Elephant Management: A Scientific Assessment for South Africa*. 1st edn. Johannesburg, SA: Wits University Press, pp. 146–205. doi: 10.18772/22008034792.14.

Kerr, T. J., de Waal, C. R., Buss, P. E., Hofmeyr, J., Lyashchenko, K. P., Miller, M. A. and Miler, M. A. (2019) 'Seroprevalence of *Mycobacterium tuberculosis* Complex in Free-ranging African Elephants (*Loxodonta africana*) in Kruger National Park, South Africa', *Journal of Wildlife Diseases*, 55(4), pp. 923–927. doi: 10.7589/2018-12-292.

Kriek, N. P. J., Areda, D. B. and Didaba, A. B. (2019) 'The Diagnosis of Bovine Tuberculosis', in Dibaba, A. B., Kriek, N. P. J., and Thoen, C. O. (eds) *Tuberculosis in Animals: An African Perspective*. Cham, Switzerland: Springer Nature Switzerland AG, pp. 171–235. doi: 10.1007/978-3-030-18690-6_9.

Lacasse, C., Terio, K., Kinsel, M. J., Farina, L. L., Travis, D. A., Greenwald, R., Lyashchenko, K. P., Miller, M. A. and Gamble, K. C. (2007) 'Two Cases of Atypical Mycobacteriosis Caused by *Mycobacterium szulgai* with Mortality in Captive African Elephants (*Loxodonta africana)*', *Journal of Zoo and Wildlife Medicine*, 38(1), pp. 101–107. doi: 10.1638/06-051.1.

Lainé, N. (2018) 'Elephant Tuberculosis as a Reverse Zoonosis: Postcolonial Scenes of Compassion, Conservation, and Public Health in Laos and France', *Medicine Anthropology Theory*, 5(3), pp. 157–176. doi: 10.17157/mat.5.3.379.

Landolfi, J. A., Mikota, S. K., Chosy, J., Lyashchenko, K. P., Giri, K., Gairhe, K. and Terio, K. A. (2010) 'Comparison of Systemic Cytokine Levels in *Mycobacterium spp*. Seropositive and Seronegative Asian Elephants (*Elephas maximus*)', *Journal of Zoo and Wildlife Medicine*, 41(3), pp. 445–455. doi: 146.232.173.180.

Landolfi, J. A., Schultz, S. A., Mikota, S. K. and Terio, K. A. (2009) 'Development and Validation of Cytokine Quantitative, Real Time RT-PCR Assays for Characterization of Asian Elephant Immune Responses', *Veterinary Immunology and Immunopathology*, 131(1–2), pp. 73–78. doi: 10.1016/j.vetimm.2009.03.012.

Landolfi, J. A., Miller, M., Maddox, C., Zuckermann, F., Langan, J. N. and Terio, K. A. (2014) 'Differences in Immune Cell Function Between Tuberculosis Positive and Negative Asian Elephants', *Tuberculosis*, 94(4), pp. 374–382. doi: 10.1016/j.tube.2014.03.001.

Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., Lopez, R., Thompson, J. D., Gibson, T. J. and Higgins, D. G. (2007) 'Clustal W and Clustal X Version 2.0', *Bioinformatics*, 23(21), pp. 2947–2948.

doi: 10.1093/bioinformatics/btm404.

Leclerc, M., Haddad, N., Moreau, R. and Thorel, M. (2000) 'Molecular Characterization of Environmental *Mycobacterium* Strains by PCR-Restriction Fragment Length Polymorphism of hsp 65, and of 16S and ITS1 rDNA', *Research in Microbiology*, 151(8), pp. 629–638. doi: 10.1016/s0923-2508(00)90129-3.

Lewerin, S. S., Olsson, S. L., Eld, K., Röken, B., Ghebremichael, S., Koivula, T., Källenius, G. and Bölske, G. (2005) 'Outbreak of *Mycobacterium tuberculosis* Infection Among Captive Asian Elephants in a Swedish Zoo', *Veterinary Record*, 156(6), pp. 171–175. doi: 10.1136/vr.156.6.171.

Livak, K. J. and Schmittgen, T. D. (2001) 'Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta CT}$ Method', *Methods*, 25, pp. 402–408. doi: 10.1006/meth.2001.1262.

Long, S. Y., Latimer, E. M. and Hayward, G. S. (2016) 'Review of Elephant Endotheliotropic Herpesviruses and Acute Hemorrhagic Disease', *Institute for Laboratory Animal Research Journal*, 56(3), pp. 283–296. doi: 10.1093/ilar/ilv041.

Lyashchenko, K. P., Greenwald, R., Esfandiari, J., Olsen, J. H., Ball, R., Dumonceaux, G., Dunker, F., Buckley, C., Richard, M., Murray, S., Payeur, J. B., Andersen, P., Pollock, J. M., Mikota, S., Miller, M., Sofranko, D. and Waters, W. R. (2006) 'Tuberculosis in Elephants: Antibody Responses to Defined Antigens of *Mycobacterium tuberculosis*, Potential for Early Diagnosis, and Monitoring of Treatment', *Clinical and Vaccine Immunology*, 13(7), pp. 722–732. doi: 10.1128/CVI.00133-06.

Lyashchenko, K. P., Greenwald, R., Esfandiari, J., Mikota, S., Miller, M., Moller, T., Vogelnest, L., Gairhe, K. P., Robbe-Austerman, S., Gai, J. and Waters, W. R. (2012) 'Field Application of Serodiagnostics To Identify Elephants with Tuberculosis Prior to Case Confirmation by Culture', *Clinical and Vaccine Immunology*, 19(8), pp. 1269–1275. doi: 10.1128/CVI.00163-12.

Lyashchenko, K. P., Gortázar, C., Miller, M. A. and Waters, W. R. (2018) 'Spectrum of Antibody Profiles in Tuberculous Elephants, Cervids, and Cattle', *Veterinary Microbiology*, 214, pp. 89–92. doi: 10.1016/j.vetmic.2017.12.013.

Maas, M., Michel, A. L. and Rutten, V. P. M. G. (2013) 'Facts and Dilemmas in Diagnosis of Tuberculosis in Wildlife', *Comparative Immunology, Microbiology and Infectious Diseases*, 36(3), pp. 269–285. doi: 10.1016/j.cimid.2012.10.010.

Maslow, J. N. and Mikota, S. K. (2015) 'Tuberculosis in Elephants-A Reemergent Disease: Diagnostic Dilemmas, the Natural History of Infection, and New Immunological Tools', *Veterinary Pathology*, 52(3), pp. 437–440. doi: 10.1177/0300985814568357.

Mikota, S. K., Peddie, L., Peddie, J., Isaza, R., Dunker, F., West, G., Lindsay, W., Scott Larsen, R., Salman, M. D., Chatterjee, D., Payeur, J., Whipple, D., Thoen, C., Davis, D. S., Sedgwick, C., Montali, R. J., Ziccardi, M. and Maslow, J. (2001) 'Epidemiology and Diagnosis of *Mycobacterium tuberculosis* in Captive Asian Elephants (*Elephas maximus*)', *Journal of Zoo and Wildlife Medicine*, 32(321), pp. 1–16. doi: 10.1638/1042-7260(2001)032[0001:EADOMT]2.0.CO;2.

Mikota, S. K. (2008) 'Review of Tuberculosis in Captive Elephants and Implications for Wild Populations', *Gajah*, 28, pp. 8–18.

Mikota, S. K. and Maslow, J. N. (2011) 'Tuberculosis at the Human-Animal Interface: An Emerging Disease of Elephants', *Tuberculosis*, 91(3), pp. 208–211. doi: 10.1016/j.tube.2011.02.007.

Miller, M. A. and Lyashchenko, K. P. (2015) 'Mycobacterial Infections in Other Zoo Animals', in Mukundan, H., Chambers, M. A., Waters, W. R., and Larsen, M. H. (eds) *Tuberculosis, Leprosy and Mycobacterial Diseases of Man and Animals: the Many Hosts of Mycobacteria*. 1st edn. Boston, USA: CAB International, pp. 277–295. doi: 10.1079/9781780643960.0277.

Miller, M. A., Finnegan, M., Storms, T., Garner, M. and Lyashchenko, K. P. (2018) 'Outbreak of *Mycobacterium tuberculosis* in a Herd of Captive Asian Elephants (*Elephas maximus*): Antemortem Diagnosis, Treatment, and Lessons Learned', *Journal of Zoo and Wildlife Medicine*, 49(3), pp. 748–754. doi: 10.1638/2017-0200.1.

Miller, M. A., Buss, P., Roos, E. O., Hausler, G., Dippenaar, A., Mitchell, E., van Schalkwyk, L., Robbe-Austerman, S., Waters, W. R., Sikar-Gang, A., Lyashchenko, K. P., Parsons, S. D. C., Warren, R. and van Helden, P. (2019) 'Fatal Tuberculosis in a Free-Ranging African Elephant and One Health Implications of Human Pathogens in Wildlife', *Frontiers in*

Veterinary Science, 6(18), pp. 1–8. doi: 10.3389/fvets.2019.00018.

Mmbaga, N. E., Munishi, L. K. and Treydte, A. C. (2017) 'Balancing African Elephant Conservation with Human Well-Being in Rombo Area, Tanzania', *Advances in Ecology*, 2017, pp. 1–9. doi: 10.1155/2017/4184261.

Obanda, V., Poghon, J., Yongo, M., Mulei, I., Ngotho, M., Waititu, K., Makumi, J., Gakuya, F., Omondi, P., Soriguer, R. C., Alasaad, S., It U, I. T., Makumi, J., Gakuya, F., Omondi, P., Soriguer, R. C. and Alasaad, D. S. (2013) 'First Reported Case of Fatal Tuberculosis in a Wild African Elephant with Past Human-Wildlife Contact', *Epidemiology and Infection*, 141(7), pp. 1476–1480. doi: 10.1017/S0950268813000022.

Olivier, T. T., Viljoen, I. M., Hofmeyr, J., Hausler, G. A., Goosen, W. J., Tordiffe, A. S. W., Buss, P., Loxton, A. G., Warren, R. M., Miller, M. A., van Helden, P. D. and Parsons, S. D. C. (2017) 'Development of a Gene Expression Assay for the Diagnosis of *Mycobacterium bovis* Infection in African Lions (*Panthera leo*)', *Transboundary and Emerging Diseases*, 64, pp. 774–781. doi: 10.1111/tbed.12436.

Ouyang, W., Rutz, S., Crellin, N. K., Valdez, P. A. and Hymowitz, S. G. (2011) 'Regulation and Functions of the IL-10 Family of Cytokines in Inflammation and Disease', *Annual Review of Immunology*, 29, pp. 71–109. doi: 10.1146/annurev-immunol-031210-101312.

Parameswaran, N. and Patial, S. (2010) 'Tumor Necrosis Factor-α Signaling in Macrophages', *Critical Review in Eukaryotic Gene Expression*, 20(2), pp. 87–103. doi: 10.1007/978-1-4020-9531-3_7.

Pari, M., Denkinger, C. M., Kik, S. V, Rangaka, M. X., Zwerling, A., Oxlade, O., Metcalfe, J. Z., Cattamanchi, A., Doawdy, D. W., Dheda, K. and Banaei, N. (2014) 'Gamma Interferon Release Assays for Detection of *Mycobacterium tuberculosis* Infection', *Clinical Microbiology Reviews*, 27(1), pp. 3–20. doi: 10.1128/CMR.00034-13.

Paudel, S., Mikota, S. K., Nakajima, C., Gairhe, K. P., Maharjan, B., Thapa, J., Poudel, A., Shimozuru, M., Suzuki, Y. and Tsubota, T. (2014) 'Molecular Characterization of *Mycobacterium tuberculosis* Isolates from Elephants of Nepal', *Tuberculosis*, 94(3), pp. 287–292. doi: 10.1016/j.tube.2013.12.008.

Paudel, S. and Toshio, T. (2016) 'Tuberculosis in Elephants: a Zoonotic Disease at the Human-

Elephant Interface.', *Japanese Journal of Zoo and Wildlife Medicine*, 21(3), pp. 65–69. doi: 10.5686/jjzwm.21.65.

Paudel, S., Villanuev, M. A., Mikota, S. K., Nakajima, C., Gairhe, K. P., Subedi, S., Rayamajhi, N., Sashika, M., Shimozuru, M., Matsuba, T., Suzuki, Y. and Tsubota, T. (2016) 'Development and Evaluation of an Interferon-γ Release Assay in Asian Elephants (*Elephas maximus*)', *Journal of Veterinary Medical Science*, 78(7), pp. 1117–1121. doi: 10.1292/jvms.15-0701.

Paudel, S. and Sreevatsan, S. (2020) 'Tuberculosis in Elephants: Origins and Evidence of Interspecies Transmission', *Tuberculosis*, 123(101962), pp. 1–6. doi: 10.1016/j.tube.2020.101962.

Perera, B. V. P., Salgadu, M. A., Gunawardena, G. S. P. de S., Smith, N. H. and Jinadasa, H. R. N. (2014) 'First Confirmed Case of Fatal Tuberculosis in a Wild Sri Lankan Elephant', *Gajah*, 41, pp. 28–31.

Radonic, A., Thulke, S., Mackay, I. M., Landt, O., Siegert, W. and Nitsche, A. (2004) 'Guideline to Reference Gene Selection for Quantitative Real-Time PCR', *Biochemical and Biophysical Research Communications*, 313, pp. 856–862. doi: 10.1016/j.bbrc.2003.11.177.

Ramani, T., Auletta, C. S., Weinstock, D., Mounho-Zamora, B., Ryan, P. C., Salcedo, T. W. and Bannish, G. (2015) 'Cytokines: The Good, the Bad, and the Deadly', *International Journal of Toxicology*, 34(4), pp. 355–365. doi: 10.1177/1091581815584918.

Rath, P. C. and Aggarwal, B. B. (1999) 'TNF-Induced Signaling in Apoptosis', *Journal of Clinical Immunology*, 19(6), pp. 350–364. doi: 10.1023/A:1020546615229.

Redford, P. S., Murray, P. J. and O'Garra, A. (2011) 'The Role of *IL-10* in Immune Regulation During *M. tuberculosis* Infection', *Mucosal Immunology*, 4(3), pp. 261–270. doi: 10.1038/mi.2011.7.

Reichardt, H. M., Tuckermann, J. P., Göttlicher, M., Vujic, M., Weih, F., Angel, P., Herrlich, P. and Schütz, G. (2001) 'Repression of Inflammatory Responses in the Absence of DNA Binding by the Glucocorticoid Receptor', *The EMBO Journal*, 20(24), pp. 7168–7173. doi: 10.1093/emboj/20.24.7168.

Rhodes, S. G., Palmer, N., Graham, S. P., Bianco, A. E., Hewinson, R. G. and Vordermeier,

H. M. (2000) 'Distinct Response Kinetics of Gamma Interferon and Interleukin-4 in Bovine Tuberculosis', *Infection and Immunity*, 68(9), pp. 5393–5400. doi: 10.1128/IAI.68.9.5393-5400.2000.

Rhodes, S. G., Gunn-Moore, D., Boschiroli, M. L., Schiller, I., Esfandiari, J., Greenwald, R. and Lyashchenko, K. P. (2011) 'Comparative Study of IFNy and Antibody Tests for Feline Tuberculosis', *Veterinary Immunology and Immunopathology*, 144, pp. 129–134. doi: 10.1016/j.vetimm.2011.07.020.

Romero-Adrian, T. B., Leal-Montiel, J., Fernandez, G. and Valecillo, A. (2015) 'Role of Cytokines and Other Factors Involved in the *Mycbacterium tuberculosis* Infection', *World Journal of Immunology*, 5(1), pp. 16–51. doi: 10.5411/wji.v5.i1.16.

Roos, E. O., Buss, P., de Klerk-Lorist, L.-M. M., Hewlett, J., Hausler, G. A., Rossouw, L., Mccall, A. J., Cooper, D., van Helden, P. D., Parsons, S. D. C. C. and Miller, M. A. (2016) 'Test Performance of Three Serological Assays for the Detection of *Mycobacterium bovis* Infection in Common Warthogs (*Phacochoerus africanus*)', *Veterinary Immunology and Immunopathology*, 182, pp. 79–84. doi: 10.1016/j.vetimm.2016.10.006.

Roos, E. O., Olea-Popelka, F., Buss, P., Hausler, G. A., Warren, R., van Helden, P. D., Parsons, S. D. C., de Klerk-Lorist, L.-M. and Miller, M. A. (2018) 'Measuring Antigen-Specific Responses in *Mycobacterium bovis*-Infected Warthogs (*Phacochoerus africanus*) Using the Intradermal Tuberculin Test', *BMC Veterinary Research*, 14(360), pp. 1–7. doi: 10.1186/s12917-018-1685-8.

Roos, E. O., Scott, L. A., Ndou, S., Olea-Popelka, F., Buss, P. E., de Klerk-Lorist, L. M., Warren, R. M., van Helden, P. D., Sylvester, T. T., Miller, M. A. and Parsons, S. D. C. (2019) 'Cytokine Gene Expression Assay as a Diagnostic Tool for Detection of *Mycobacterium bovis* Infection in Warthogs (*Phacochoerus africanus*)', *Scientific Reports*, 9(1), pp. 1–7. doi: 10.1038/s41598-019-53045-0.

Rosen, L. E., Hanyire, T. G., Dawson, J., Foggin, C. M., Michel, A. L., Huyvaert, K. P., Miller, M. A. and Olea-Popelka, F. J. (2018) 'Tuberculosis Serosurveillance and Management Practices of Captive African Elephants (*Loxodonta africana*) in the Kavango-Zambezi Transfrontier Conservation Area', *Transboundary and Emerging Diseases*, 65(2), pp. e344–e354. doi: 10.1111/tbed.12764.

Sabat, R., Grütz, G., Warszawska, K., Kirsch, S., Witte, E., Wolk, K. and Geginat, J. (2010) 'Biology of Interleukin-10', *Cytokine and Growth Factor Reviews*, 21, pp. 331–344. doi: 10.1016/j.cytogfr.2010.09.002.

Sach, F., Dierenfeld, E. S., Langley-Evans, S. C., Watts, M. J. and Yon, L. (2019) 'African Savanna Elephants (*Loxodonta africana*) as an Example of a Herbivore Making Movement Choices Based on Nutritional Needs', *PeerJ*, 7(e6260), pp. 1–27. doi: 10.7717/peerj.6260.

de Sales, A. R., Anastácio, S. R. S. and Pereira, M. J. (2020) 'The African Elephant (*Loxodonta africana*): Mini-Review of an Endangered Species', *Natural Resources*, 11, pp. 317–350. doi: 10.4236/nr.2020.118019.

Sanjabi, S., Zenewicz, L. A., Kamanaka, M. and Flavell, R. A. (2009) 'Anti- and Pro-inflammatory Roles of TGF-β, IL-10, and IL-22 In Immunity and Autoimmunity', *Current Opinion in Pharmacology*, 9(4), pp. 447–453. doi: 10.1016/j.coph.2009.04.008.

Sanjabi, S., Oh, S. A. and Li, M. O. (2017) 'Regulation of the Immune Response by TGF-β: From Conception to Autoimmunity and Infection', *Cold Spring Harbor Perspectives in Biology*, 9(6), pp. 1–34. doi: 10.1101/cshperspect.a022236.

Saraiva, M., Vieira, P. and O'Garra, A. (2019) 'Biology and Therapeutic Potential of Interleukin-10', *Journal of Experimental Medicine*, 217(1), pp. 1–19. doi: 10.1084/jem_20190418.

Schlossberg, S., Chase, M. J. and Sutcliffe, R. (2019) 'Evidence of a Growing Elephant Poaching Problem in Botswana', *Current Biology*, 29(13), pp. 2222–2228. doi: https://doi.org/10.1016/j.cub.2019.05.061.

Schmittgen, T. D. and Livak, K. J. (2008) 'Analyzing Real-Time PCR Data by the Comparative CT Method', *Nature Protocols*, 3(6), pp. 1101–1108. doi: 10.1038/nprot.2008.73.

Shaw, D. M., Merien, F., Braakhuis, A. and Dulson, D. (2018) 'T-cells and their Cytokine Production: The Anti-Inflammatory and Immunosuppressive Effects of Strenuous Exercise', *Cytokine*, 104, pp. 136–142. doi: 10.1016/j.cyto.2017.10.001.

Songthammanuphap, S., Puthong, S., Pongma, C., Buakeaw, A., Prammananan, T., Warit, S., Tipkantha, W., Kaewkhunjob, E., Yindeeyoungyeon, W. and Palaga, T. (2020) 'Detection of

Mycobacterium tuberculosis Complex Infection in Asian Elephants (*Elephas maximus*) Using an Interferon Gamma Release Assay in a Captive Elephant Herd', *Scientific Reports*, 10(14551), pp. 1–10. doi: 10.1038/s41598-020-71099-3.

Sreekumar, E., Janki, M. B. V, Arathy, D. S., Hariharan, R., Avinash Premraj, C. and Rasool, T. J. (2007) 'Molecular Characterization and Expression of Interferon-γ of Asian elephant (Elephas maximus)', *Veterinary Immunology and Immunopathology*, 118, pp. 75–83. doi: 10.1016/j.vetimm.2007.04.012.

Thacker, T. C., Palmer, M. V and Waters, W. R. (2006) 'Correlation of Cytokine Gene Expression with Pathology in White-Tailed Deer (*Odocoileus virginianus*) Infected with *Mycobacterium bovis*', *Clinical and Vaccine Immunology*, 13(6), pp. 640–647. doi: 10.1128/CVI.00024-06.

Thoen, C O and Barletta, R. G. (2006) 'Pathogenesis of *Mycobacterium bovis*', in Thoen, Charles O, Steele, J. H., and Gilsdorf, M. J. (eds) *Mycobacterium bovis Infection in Animals and Humans*. 2nd edn. Iowa, USA: Blackwell Publishing Ltd, pp. 18–33. doi: 10.1002/9780470344538.ch4.

Toossi, Z. and Ellner, J. J. (1998) 'The Role of TGFb in the Pathogenesis of Human Tuberculosis', *Clinical Immunology and Immunopathology*, 87(2), pp. 107–114. doi: 10.1006/clin.1998.4528.

Travis, M. A. and Sheppard, D. (2014) 'TGF-β Activation and Function in Immunity', *Annual Review of Immunology*, 32, pp. 51–82. doi: 10.1146/annurev-immunol-032713-120257.

United States Animal Health Association (USAHA) Elephant Tuberculosis Subcommittee (2010) 'Guidelines for the Control of Tuberculosis', in *Elephant TB Guidelines*, pp. 1–53. Available at: https://www.aphis.usda.gov/animal_welfare/downloads/elephant/TB-Guidelines-Final-8-November-2010.pdf.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A. and Speleman, F. (2002) 'Accurate Normalization of Real-Time Quantitative RT-PCR Data by Geometric Averaging of Multiple Internal Control Genes', *Genome Biology*, 3(7), pp. 1–12. doi: 10.1186/gb-2002-3-7-research0034.

VanGuilder, H. D., Vrana, K. E. and Freeman, W. M. (2008) 'Twenty-Five Years of

Quantitative PCR for Gene Expression Analysis', *BioTechniques*, 44(5), pp. 619–626. doi: 10.2144/000112776.

Verma-Kumar, S., Abraham, D., Dendukuri, N., Cheeran, J. V., Sukumar, R. and Balaji, K. N. (2012) 'Serodiagnosis of Tuberculosis in Asian Elephants (*Elephas maximus*) in Southern India: A Latent Class Analysis', *PLoS ONE*, 7(11), p. e49548. doi: 10.1371/journal.pone.0049548.

Walzl, G., Ronacher, K., Hanekom, W., Scriba, T. J. and Zumla, A. (2011) 'Immunological Biomarkers of Tuberculosis', *Nature Reviews Immunology*, 11, pp. 343–354. doi: 10.1038/nri2960.

Wang, H., Czura, C. J. and Tracey, K. J. (2003) 'Tumor Necrosis Factor', in Thomson, A. W. and Lotze, M. T. (eds) *The Cytokine Handbook*. 4th edn. London, UK: Elsevier Science Ltd, pp. 837–860. doi: 10.1016/B978-012689663-3/50018-1.

Warren, R. M., Gey Van Pittius, N. C., Barnard, M., Hesseling, A., Engelke, E., de Kock, M., Gutierrez, M. C., Chege, G. K., Victor, T. C., Hoal, E. G. and Van Helden, P. D. (2006) 'Differentiation of *Mycobacterium tuberculosis* Complex by PCR Amplification of Genomic Regions of Difference', *International Journal of Tuberculosis and Lung Disease*, 10(7), pp. 818–822.

World Organisation for Animal Health (OIE) (2018) 'Bovine Tuberculosis', in *Manual of Diagnostic Tests and Vaccines for Terrestial Animals*. 8th edn, pp. 1058–1074. Available at: https://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/3.04.06_BOVINE_TB.pdf.

Zachariah, A., Pandiyan, J., Madhavilatha, G. K., Mundayoor, S., Chandramohan, B., Sajesh, P. K., Santhosh, S. and Mikota, S. K. (2017) '*Mycobacterium tuberculosis* in Wild Asian Elephants, Southern India', *Emerging Infectious Diseases*, 23(3), pp. 504–506. doi: 10.3201/eid2303.161741.

Zuniga, J., Torres-Garcia, D., Santos-Mendoza, T., Rodriquez-Reyna, T. S., Granados, J. and Yunis, E. J. (2012) 'Cellular and Humoral Mechanisms Involved in the Control of Tuberculosis', *Clinical and Developmental Immunology*, 2012(193923), pp. 1–18. doi: 10.1155/2012/193923.

Appendices

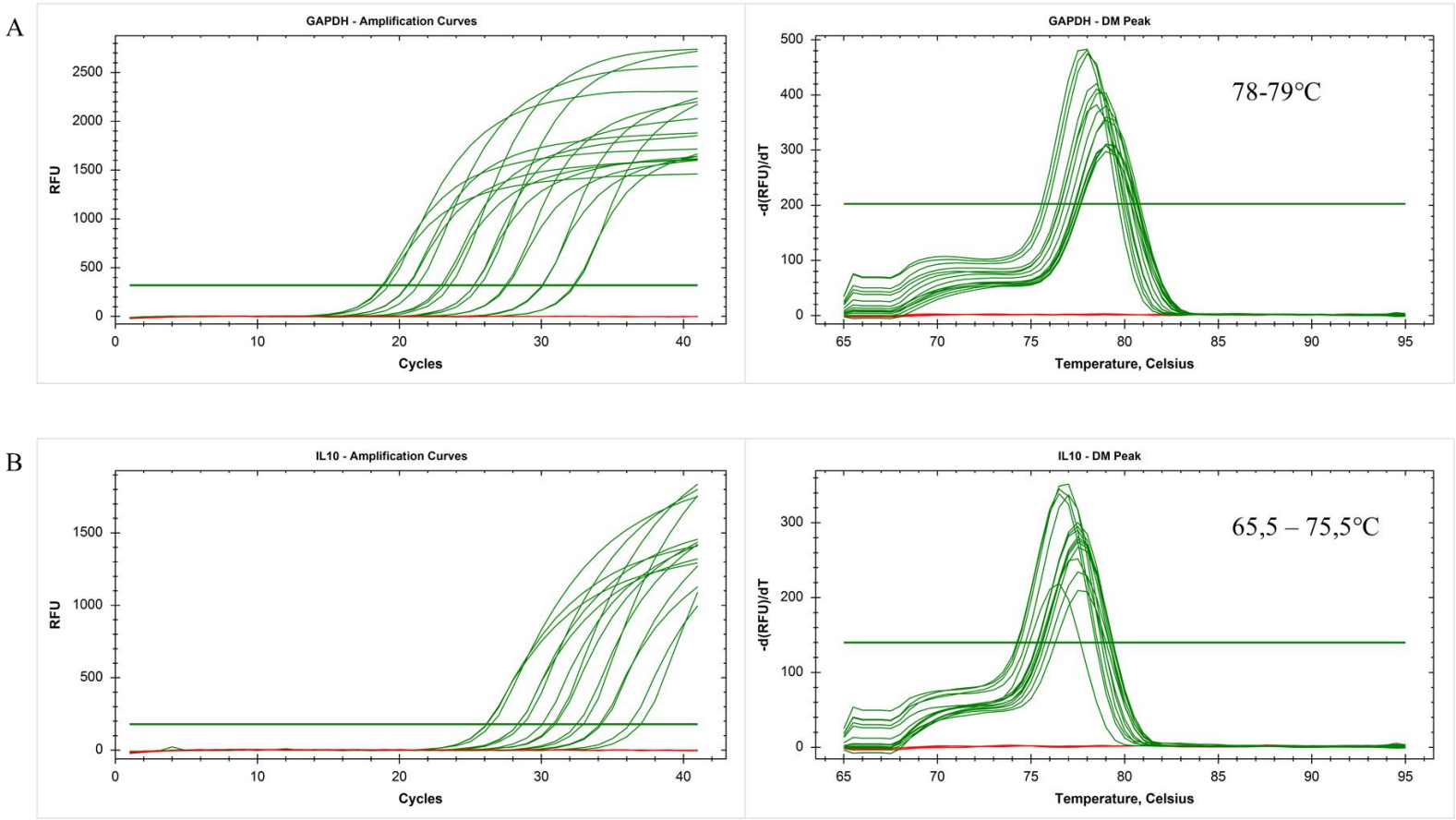
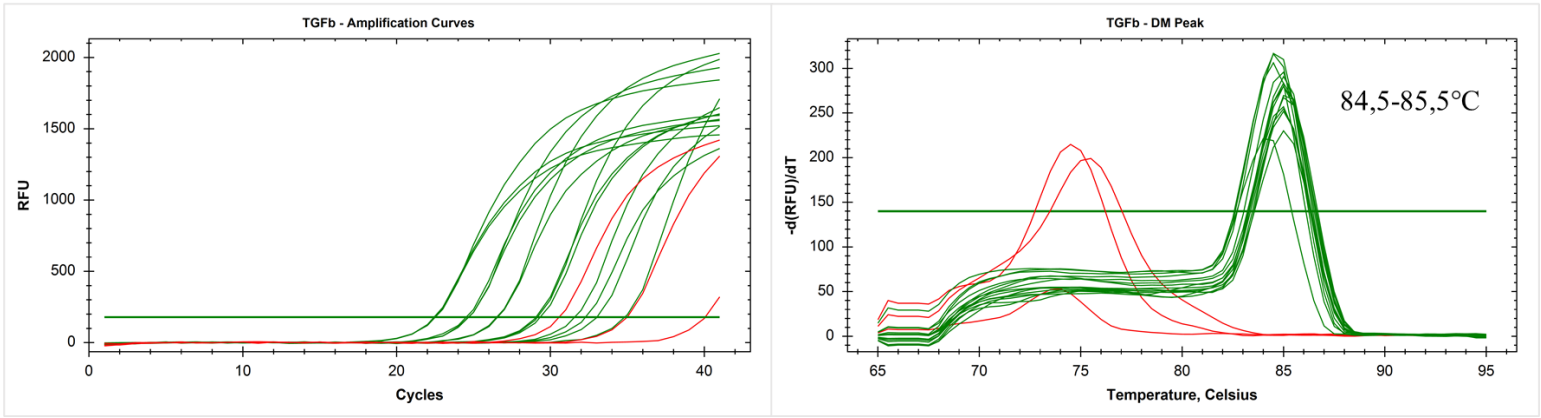


Figure 1: Amplification curves and derivative melt peaks (DM) of *GAPDH* (A), *IL10* (B), *TGFβ* (C) and *TNF* (D) following amplification of a five-fold serial dilution, with an estimated cDNA range of 1000 to 0.064 ng. Each dilution point was run in triplicate (indicated in green) and amplification efficiency (E) determined as previously described. No-template controls are indicated in green. The derivative melt peak (M) temperature range (°C) of each gene is also indicated.

C



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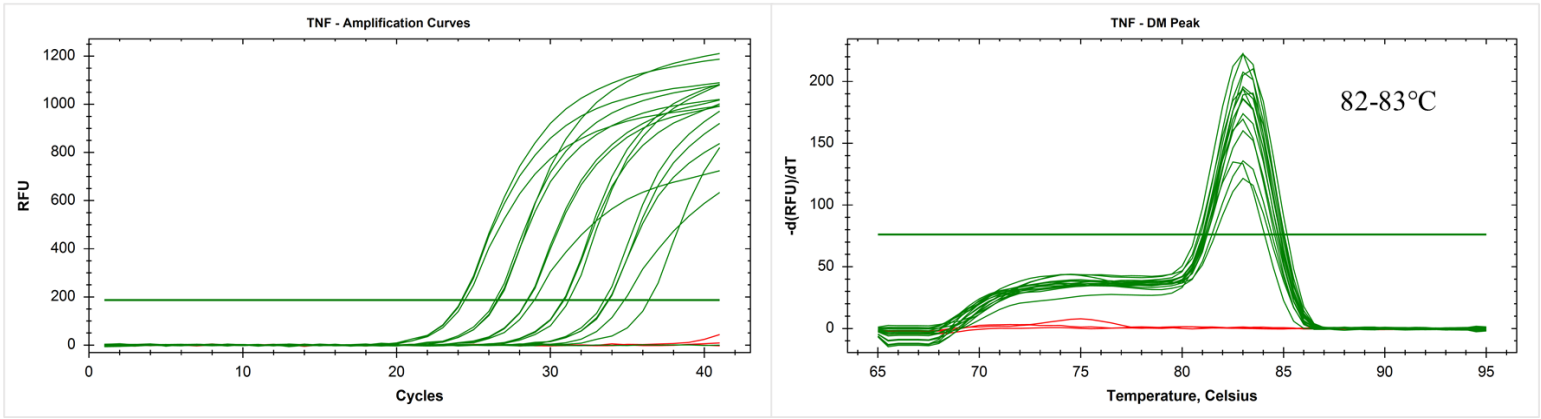

Figure 1 (Continued): Amplification curves and derivative melt peaks (DM) of *GAPDH* (A), *IL10* (B), *TGFβ* (C) and *TNF* (D) following amplification of a five-fold serial dilution, with an estimated cDNA range of 1000 to 0.064 ng. Each dilution point was run in triplicate (indicated in green) and amplification efficiency (E) determined as previously described. No-template controls are indicated in green. The derivative melt peak (M) temperature range (°C) of each gene is also indicated.

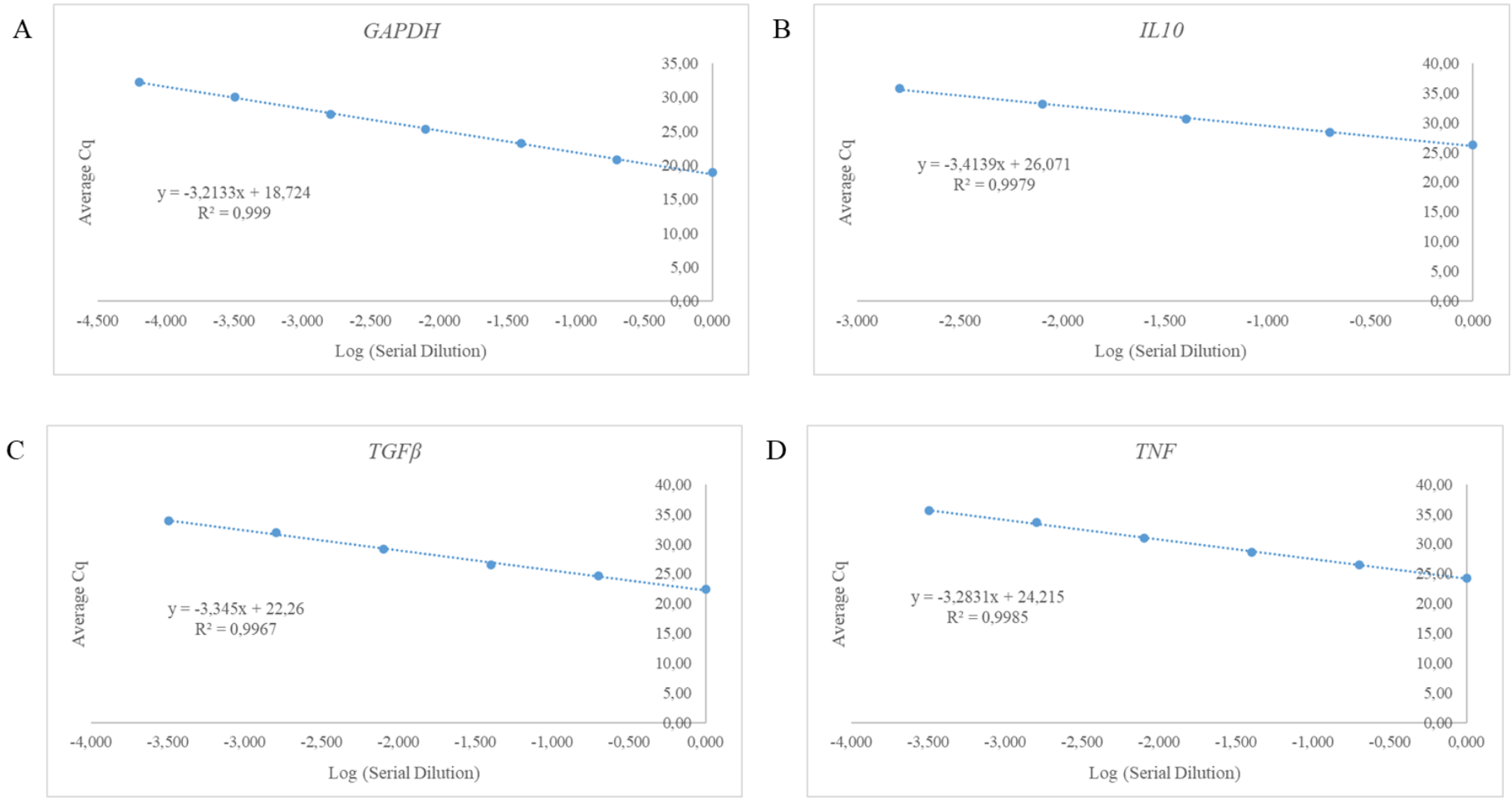

Figure 2: Standard curves of *GAPDH* (A), *IL10* (B), *TGFβ* (C) and *TNF* (D) used to determine amplification efficiencies (E) as previously described (Livak and Schmittgen, 2001), following amplification of a five-fold serial dilution, with an estimated cDNA range of 1000 to 0.064 ng. Amplification efficiencies were calculated as $E = 10^{(-1/slope)} \times 100$.

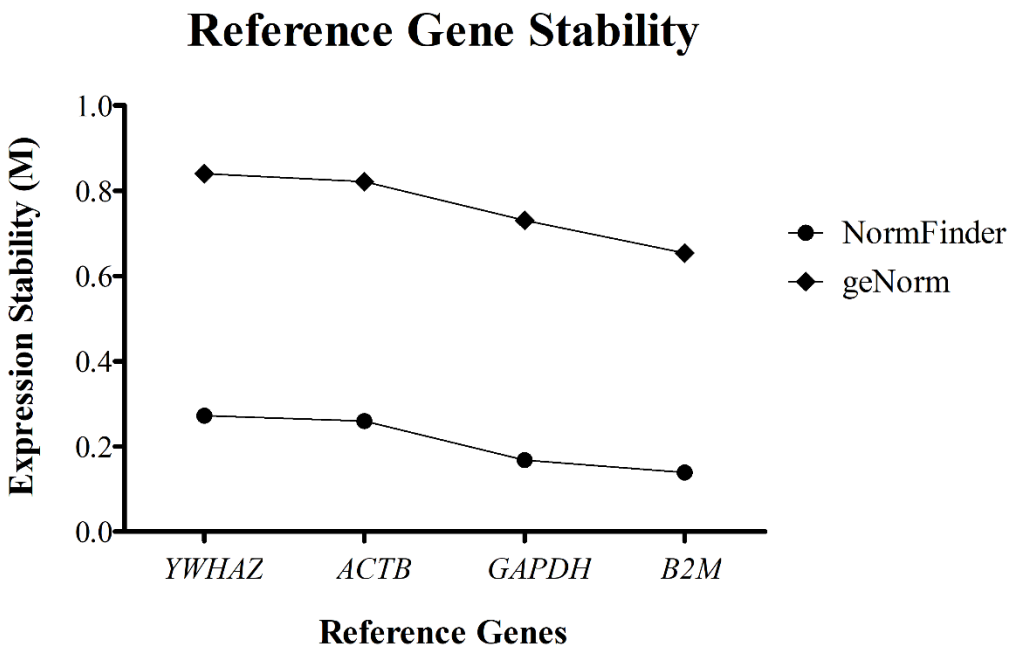

Figure 3: Expression stability of candidate reference genes as calculated using the geNorm applet in Microsoft Excel (Vandesompele *et al.*, 2002) and the NormFinder Excel Add-In (Andersen, Jensen and Ørntoft, 2004). Whole blood from three African elephants stimulated with PBS and 10 µg/mL was used to determine stability values.